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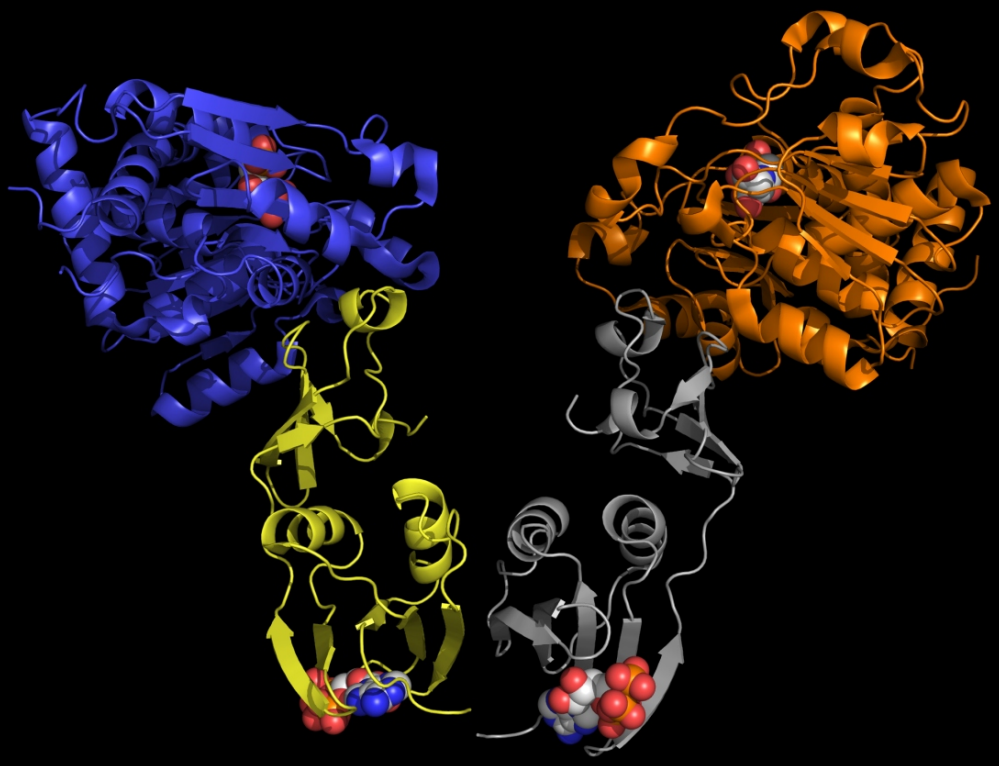
Understanding ENZYMES

An Introductory Text

Aditya Arya Amit Kumar Jayanti Jha



First Edition

Understanding

ENZYMES

An Introductory Text

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Although utmost care has been taken to avoid any errors in the preparation of answers, however in case of any discrepancies or loss of any kind due to incorrect answers or solutions, authors or publishers shall not be responsible. Also, the concepts of enzymology, mentioned in the book are not for clinical advice or suggestions.

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To

Our Loving Parents

ABOUT THE AUTHORS

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Dr. Amit is Ph.D. in the area of Marine Molecular Ecology from Stazione Zoologica Anton Dohrn, Naples, Italy. He has published 15+ research papers and book chapters in peer-reviewed international journals/books. He holds expertise in oxidative and nitrosative stress enzymology of marine plants. Dr. Amit is also the recipient of prestigious international research fellowships and grants in the domain of marine Ecology. He was trained in various reputed research laboratories in Europe, including Verona University, Antwep University, Roscoff Marine Station, and University of Gothenburg. He has delivered several scientific talks at national and international platforms. He is an expert open water diver and participated in several scientific cruise expeditions.

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PREFACE

Enzymes represent one of the most fascinating and most meaningful entities in the biological world. In fact, in absence of enzymes, nothing would have been possible, neither evolution of inorganic compounds to life, nor technological advancements in various phases of industrialization. Perhaps, the importance of enzyme is also reflected in the number of Nobel prizes awarded in this domain and the curriculum of Life Sciences courses in most of the universities, which also contain enzymology as an independent subject. Enzymes as a biological catalysts have been known to all of us since our primary education. Perhaps, we all know the fact that enzymes speed up the biological reactions by lowering the activation energy, but do we really understand, how does this decrease happen? What is the importance of activation energy? What is the difference between transition state and intermediate state? Such questions often remain unanswered for a larger clad of undergraduates. These unclear terms and unanswered questions in enzymology, challenge the learning of enzymes at the higher educational degree and diploma courses. This book has been particularly written to augment the understanding of enzymology in graduate and postgraduate students, but not limited to and can be used by school and college teachers, researchers and professors as a resource book. This book consists of 10 chapters which include a detailed discussion of key concepts of enzymology, enzyme kinetics, modes of enzyme regulation, isozymes, enzyme technology, and applications. The balanced text, well-illustrated concepts and organized stepwise ascent of the topics are key features of this book. Besides fundamentals, this books also covers some of the emerging areas of enzymology such as abzymes, ribozymes, synzyme, pseudozymes and nanozymes. Difficult concepts have been simplified with analogy and examples. A number of practice questions have also been included throughout the text. Tables of comparison, key points, and examples have been extensively created, some of them also constitute useful appendices in the book.

The simplicity of text, illustration, and analogies with real-life conditions and concise nature makes this book different from existing texts in the domain. Nevertheless, the information is updated to the contemporary research and brings in rather new concepts of the enzymology that have been added in present decade. The content of the book matches with the enzymology courses of several universities in India and abroad. Besides regular curriculum of several life science courses in universities, this books has also been developed with a consideration of topics and pattern of competitive exams for MSc entrance and research fellowships (JNU, BHU, JAM, DU, CSIR, GATE GRE etc.). A brief booklet of practice questions will soon follow this book in next edition. The authors of this book have extensive experience of research in enzymes and teaching enzymology with proven and admired competence in the field.

We acknowledge the efforts and suggestions of Subhojit Paul, Anamika Gangwar, Shikha Jain, Pooja, and Nassruddin. Dr. Aditya also thank his teachers Prof. G.S Selvam, Prof. P. Palinivelu and Prof. CK Shrotri for introducing enzymology as an interesting subject and he also thanks, Prof. Mainak Das Dr. Yasmin Ahmad and Dr. Kalpana Bhargava for being excellent mentors during his Ph.D.. The moral support of family members and friends was also an essential driving force for the completion of this book. We also sincerely acknowledge our students for being the source of inspiration. Any constructive suggestion or improvements are always welcome.

Authors
14th Feb, 2018

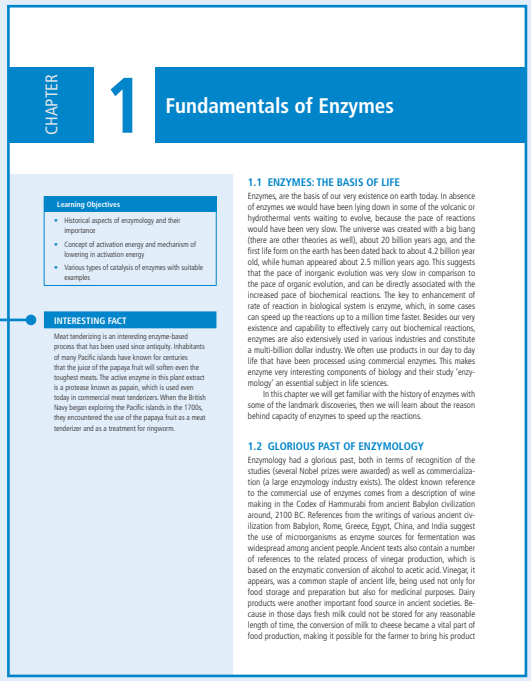
CONTENTS

A NOTE TO READERS

This book "Understanding enzymes: An Introductory Text" has been written to meet the needs of a beginner in enzymology. This book contains recent updates and latest information on enzymes along with well-established concepts of enzymes explained in a lucid manner with illustrations and analogies. The book begins with a very basic introduction on enzymes, including glorious past of enzymology, a context of chronological discoveries in enzymology and a simplified description of the reason behind the catalytic power of enzymes. Next few chapters are based on classification and kinetics of enzymes. Kinetics has been divided across two chapters, first one is essential kinetics with a basic understanding of chemical kinetics, Michaelis-Menten kinetics etc., while second chapter is about on a more advanced version of kinetics including pre-steady-state kinetics, allosteric kinetics, and bisubstrate kinetics. A large emphasis has been given to the various type of graphical presentations of kinetic data and their relation with mathematical equations. Enzyme regulation has been explained to a greater extent with sufficient examples and clarity. An important chapter on non-canonical enzymes brings the latest updates in the domain of enzymology. The latter part of this book focuses on enzyme technology and applications of enzymes in industries and various other commercial sectors, which makes this book useful for aspirants of enzyme engineering and other technical streams as well. A number of tables of comparison, classification charts and examples have been provided wherever required to improve the learning experience for a beginner. Some special components have been added to each chapter to include additional information, practice questions and practice questions and side notes.

INTERESTING FACT

Each chapter contains an interesting fact that provides a unique feature of enzymes in context of the given chapter. Sometimes, interesting facts also highlight the contemporary developments in enzymology or extraordinary potentials of enzymes in life.



CHAPTER 1 Fundamentals of Enzymes

Learning Objectives

- Historical aspects of enzymology and their importance
- Concept of activation energy and mechanism of lowering in activation energy
- Various types of catalysis of enzymes with suitable examples

INTERESTING FACT

Meat tenderizing is an interesting enzyme-based process that has been used since antiquity. Inhabitants of many Pacific islands have known for centuries that the juice of the papaya fruit will soften even the toughest meats. The active enzyme in this plant extract is a protease known as papain, which is used even today in commercial meat tenderizers. When the British Navy began exploring the Pacific islands in the 1700s, they encountered the use of the papaya fruit as a meat tenderizer and as a treatment for ringworm.

1.1 ENZYMES: THE BASIS OF LIFE

Enzymes, are the basis of our very existence on earth today. In absence of enzymes we would have been lying down in some of the volcanic or hydrothermal vents waiting to evolve, because the pace of reactions would have been very slow. The universe was created with a big bang (there are other theories as well), about 20 billion years ago, and the first life form on the earth has been dated back to about 4.2 billion year old, while human appeared about 2.5 million years ago. This suggests that the pace of inorganic evolution was very slow in comparison to the pace of organic evolution, and can be directly associated with the increased pace of biochemical reactions. The key to enhancement of rate of reaction in biological system is enzyme, which, in some cases can speed up the reactions up to a million time faster. Besides our very existence and capability to effectively carry out biochemical reactions, enzymes are also extensively used in various industries and constitute a multi-billion dollar industry. We often use products in our day to day life that have been processed using commercial enzymes. This makes enzyme very interesting components of biology and their study 'enzymology' an essential subject in life sciences.

In this chapter we will get familiar with the history of enzymes with some of the landmark discoveries, then we will learn about the reason behind capacity of enzymes to speed up the reactions.

1.2 GLORIOUS PAST OF ENZYMOLOGY

Enzymology had a glorious past, both in terms of recognition of the studies (several Nobel prizes were awarded) as well as commercialization (a large enzymology industry exists). The oldest known reference to the commercial use of enzymes comes from a description of wine making in the Codex of Hammurabi from ancient Babylon civilization around, 2100 BC. References from the writings of various ancient civilization from Babylon, Rome, Greece, Egypt, China, and India suggest the use of microorganisms as enzyme sources for fermentation was widespread among ancient people. Ancient texts also contain a number of references to the related process of vinegar production, which is based on the enzymatic conversion of alcohol to acetic acid. Vinegar, it appears, was a common staple of ancient life, being used not only for food storage and preparation but also for medicinal purposes. Dairy products were another important food source in ancient societies. Because in those days fresh milk could not be stored for any reasonable length of time, the conversion of milk to cheese became a vital part of food production, making it possible for the farmer to bring his product

cells were not required for carrying out metabolic processes such as fermentation. Instead, there must be some small entities capable of converting sugar to alcohol. These entities were enzymes. Buchner's accidental discovery won him the 1907 Nobel Prize in chemistry.

The next breakthrough in enzymology came in 1926 when J. B. Sumner from Cornell University, U.S.A., isolated, purified and also successfully crystallized the enzyme **urease** from jack bean. (Urease breaks down urea and produces ammonia and carbon dioxide). He found that the urease crystals are purely made of proteins and hence he reported that "enzymes are nothing but proteins". But his conclusion was vehemently opposed by the well-known German biochemist Richard Willstätter, (Nobel laureate), who insisted that enzymes are nothing but 'low molecular weight organic compounds' and the protein crystals that were found in the urease preparation could be 'impurities'. For their seminal contributions to enzymology, Sumner and Northrop were awarded Nobel Prize in 1935.

The above discoveries proved beyond doubt that enzymes are nothing but proteins and are found in all living cells. Therefore, it was imperative that only living organisms could make such sophisticated enzyme molecules. However, development of solid-phase peptide synthesis by R. B. Merrifield and his group in 1964 paved the way for laboratory synthesis of enzymes for the first time. The first enzyme that was assembled on a solid phase matrix was the pancreatic ribonuclease, which contains 124 amino acids. Interestingly, the laboratory-assembled enzyme exhibited identical properties as those of the natural enzyme. Therefore, this discovery added one more dimension to enzymology and led to the synthesis of tailor-made synthetic enzymes, which are known as **Synzymes.** Later in 1969, Jenks, suggested that generating antibodies raised against a stable analogue of the transition state of the reaction that one wished to-catalyze, one could obtain antibodies with catalytic activity, and such antibodies were called **Abzymes**. The discovery of new types of enzymes, viz., the restriction enzymes and ligases in 1960s paved way for the modern discipline, the recombinant DNA technology. The discovery of restriction endonucleases by W. Arber and D. Nathan and Peter Lobbund and Dale Kaiser's work on enzymatic joining of DNA fragments led to the construction of the first recombinant DNA molecule in 1980s. Arber and Nathan were awarded Nobel Prize for their discovery of the restriction endonucleases in 1978. Thus, the successful application in construction of recombinant DNA molecules paved the way for today's new and vibrant branch of biology, the Biotechnology.

The belief that "all enzymes are proteins" was shattered in 1982 when Thomas R. Cech and Sidney Altman independently discovered that certain RNA molecules also exhibited catalytic properties like enzymes. The discovery of catalytic RNAs forced proteins to relinquish their status as the only molecules that can catalyze chemical reactions in living cells. These ribonucleic acid enzymes are called as **Ribozymes.** For this important discovery in enzymology, they were also awarded Noble Prize in 1989. Enzymology and enzyme technology witnessed great strides from 1990 onwards, with the introduction of newer techniques in the field of genetic engineering. These techniques enable direct manipulation of genes, which code for these enzymes. One of the techniques, known as the site-directed mutagenesis, developed by Michael Smith has been employed for precisely manipulating the gene of any enzyme even at one nucleotide level (which alters one amino acid) and study its effect on the properties of the new, mutant enzyme. For this discovery, he was awarded Noble Prize in 1996. Genetic engineering techniques are applied to make chimeric enzymes with multifunctional activities. Genetic engineering techniques have also made possible to purify enzymes and proteins in one step by adding special purification tags at the end of their genes. From 1997 onwards, the DNA molecules, which were once thought to be passive molecules, have also joined the bandwagon. It has been reported that some DNA molecules also exhibit catalytic properties similar to RNA molecules and thus giving birth to a new era of Deoxyribo nucleic acid enzymes (DNAzymes). Recently zeolites (silicate-based inorganic crystalline materials) are also found to mimic natural enzymes. In the 21st century, the list may include computer assisted designer enzymes, complex carbohydrates and lipoproteins, inorganic

Note

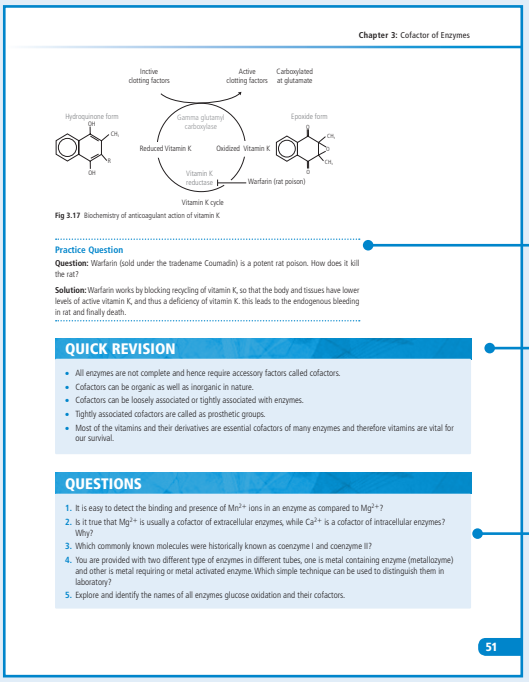
Canavalia ensiformis, or jack bean, is a legume which is used for animal fodder and human nutrition, especially in Brazil where it is called feijão-de-porco ("pig bean"). It is also the source of concanavalin A.
it cannot be used in fodder mixtures containing urea, since it contains large quantities of the enzyme urease, which liberates harmful ammonia from urea.

Jack Beans

The spontaneous cleavage of a peptide bond would take 400 years at room temperature and phospho-monoester hydrolysis, routinely breaking up ATP to release energy, would take about a million years in the absence of the enzymes.

SIDE NOTE

A number of side notes and comments have been added to each chapter to clarify some difficult terms or to emphasize certain terms and statements about the enzymes. Confusing terms, ambiguous usage of terms and definitions have also been included in side notes.



Chapter 3: Cofactor of Enzymes

Inctive clotting factors
Active clotting factors
Carboxylated at glutamate
Hydroquinone form
Gamma glutamyl carboxylase
Epoxide form
Reduced Vitamin K
Oxidized Vitamin K
Vitamin K reductase
Warfarin (rat poison)
Vitamin K cycle

Fig 3.17 Biochemistry of anticoagulant action of vitamin K

Practice Question

Question: Warfarin (sold under the tradename Coumadin) is a potent rat poison. How does it kill the rat?

Solution: Warfarin works by blocking recycling of vitamin K, so that the body and tissues have lower levels of active vitamin K, and thus a deficiency of vitamin K. this leads to the endogenous bleeding in rat and finally death.

QUICK REVISION

- All enzymes are not complete and hence require accessory factors called cofactors.
- Cofactors can be organic as well as inorganic in nature.
- Cofactors can be loosely associated or tightly associated with enzymes.
- Tightly associated cofactors are called as prosthetic groups.
- Most of the vitamins and their derivatives are essential cofactors of many enzymes and therefore vitamins are vital for our survival.

QUESTIONS

1. It is easy to detect the binding and presence of Mn^{2+} ions in an enzyme as compared to Mg^{2+}?
2. Is it true that Mg^{2+} is usually a cofactor of extracellular enzymes, while Ca^{2+} is a cofactor of intracellular enzymes? Why?
3. Which commonly known molecules were historically known as coenzyme I and coenzyme II?
4. You are provided with two different type of enzymes in different tubes, one is metal containing enzyme (metallozyme) and other is metal requiring or metal activated enzyme. Which simple technique can be used to distinguish them in laboratory?
5. Explore and identify the names of all enzymes glucose oxidation and their cofactors.

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PRACTICE QUESTIONS

Sufficiently large number of practice questions have been added at each point, wherever the need of practice is felt based on the needs of an enzymology beginner. These questions are directly related to the context at the specific place and most questions are either solved or provided with an elaborate hint for solving.

QUICK REVISION

The quick revision provides a glimpse of the complete chapter in just a few lines and leaves readers with a very quick recap of the concepts that were provided in the chapters. It is also helpful to obtain a bird's eye view of each chapter.

QUESTIONS

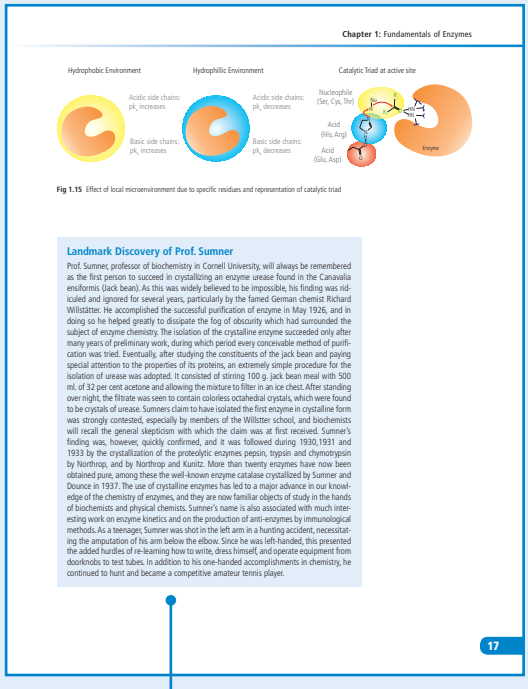
Five questions per chapter have also been included at the end of each chapter based on an overall understanding of a topic, through problems and numerical ability. Although readers are advised to spend time in solving these question, the solutions and hints have also been provided at the end of the book.

APPENDICES

This book also contains a number of appendices with some of the highly useful information for readers especially a quick reference guide for drugs against various enzymes, regulatory enzymes of various pathways and their regulators and commercial sources of enzymes.

APPENDIX 1 **Recommended Symbols in Enzymology**

Symbol	Description	Unit
[A]	Concentration of substrate A	mol dm^{-3}
$[A]_{0.5}$ or $[A]_{1/2}$	Value of [A] at which **v** = 0.5 **V**	mol dm^{-3}
[B]	Concentration of substrate B	mol dm^{-3}
$[E]_t$ (or $[E]_0$ or $[E]_{total}$)	Stoichiometric concentration of active centres	mol dm^{-3}
h (or n_H)	Hill coefficient	dimensionless
[I]	Concentration of inhibitor I	mol dm^{-3}
k	Rate constant of any order **n**	(mol dm^{-3})$^{1-n}$ s^{-1}
k	pH-independent value of **k**	as **k**
k_{app}	Apparent value of **k**	as **k**
k_A, k_B	Specificity constants for A, B	mol^{-1} dm^3 s^{-1}
k_A^2 (etc.)	Reciprocal Dalziel coefficient	(various)
k_{cat} (or k_2)	Catalytic constant	s^{-1}
ki, **k-i**	Forward and reverse rate constants respectively for i^{th} step	as **k**
K_i	Inhibition constant (inhibition type unspecified)	mol dm^{-3}
K_{iA}	Inhibition constant for A	mol dm^{-3}
K_{ic}	Competitive inhibition constant	mol dm^{-3}
k_{ij}	Rate constant for step from E_i to E_j	as **k**
K_{iu}	Uncompetitive inhibition constant	mol dm^{-3}
K_m	Michaelis constant (or Michaelis concentration)	mol dm^{-3}
K_{mA}	Michaelis constant for A	mol dm^{-3}
K_s	Substrate dissociation constant	mol dm^{-3}
K_{sA}	Value of K_s for substrate A	mol dm^{-3}
k_3 (or k_{cat})	Catalytic constant	s^{-1}
[Q]	Concentration of activator Q	mol dm^{-3}
t	Time	s
v	Rate (or velocity) of reaction	mol dm^{-3} s^{-1}
V (or V_{max})	Limiting rate (or maximum rate, or maximum velocity)	mol dm^{-3} s^{-1}
v_A	Rate of consumption of A	mol dm^{-3} s^{-1}
vi, **v-i**, **vij**	Chemical flux (or chemiflux) through step with rate constant **ki**, **k-i**, **kij**	mol dm^3 s^{-1}



Chapter 1: Fundamentals of Enzymes

Hydrophobic Environment
Hydrophilic Environment
Catalytic Triad at active site
Acidic side chains: pk$_a$ increases
Basic side chains: pk$_a$ increases
Acidic side chains: pk$_a$ decreases
Basic side chains: pk$_a$ decreases
Nucleophile (Ser, Cys, Thr)
Acid (His, Arg)
Acid (Glu, Asp)
Enzyme

Fig 1.15 Effect of local microenvironment due to specific residues and representation of catalytic triad

Landmark Discovery of Prof. Sumner

Prof. Sumner, professor of biochemistry in Cornell University, will always be remembered as the first person to succeed in crystallizing an enzyme urease found in the Canavalia ensiformis (Jack bean). As this was widely believed to be impossible, his finding was ridiculed and ignored for several years, particularly by the famed German chemist Richard Willstätter. He accomplished the successful purification of enzyme in May 1926, and in doing so he helped greatly to dissipate the fog of obscurity which had surrounded the subject of enzyme chemistry. The isolation of the crystalline enzyme succeeded only after many years of preliminary work, during which period every conceivable method of purification was tried. Eventually, after studying the constituents of the jack bean and paying special attention to the properties of its proteins, an extremely simple procedure for the isolation of urease was adopted. It consisted of stirring 100 g. jack bean meal with 500 ml. of 32 per cent acetone and allowing the mixture to filter in an ice chest. After standing over night, the filtrate was seen to contain colorless octahedral crystals, which were found to be crystals of urease. Sumners claim to have isolated the first enzyme in crystalline form was strongly contested, especially by members of the Willstter school, and biochemists will recall the general skepticism with which the claim was at first received. Sumner's finding was, however, quickly confirmed, and it was followed during 1930,1931 and 1933 by the crystallization of the proteolytic enzymes pepsin, trypsin and chymotrypsin by Northrop, and by Northrop and Kunitz. More than twenty enzymes have now been obtained pure, among these the well-known enzyme catalase crystallized by Sumner and Dounce in 1937. The use of crystalline enzymes has led to a major advance in our knowledge of the chemistry of enzymes, and they are now familiar objects of study in the hands of biochemists and physical chemists. Sumner's name is also associated with much interesting work on enzyme kinetics and on the production of anti-enzymes by immunological methods. As a teenager, Sumner was shot in the left arm in a hunting accident, necessitating the amputation of his arm below the elbow. Since he was left-handed, this presented the added hurdles of re-learning how to write, dress himself, and operate equipment from doorknobs to test tubes. In addition to his one-handed accomplishments in chemistry, he continued to hunt and became a competitive amateur tennis player.

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Boxes

A more detailed version of few topics that remain within the scope of the specific chapter and need emphasis have been included in separate boxes. These topics represent some of the most important discoveries in enzymology, recent developments or sometimes a few techniques related to the context.

CHAPTER 1

Fundamentals of Enzymes

Learning Objectives

- Historical aspects of enzymology and their importance
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to distant markets in an acceptable form. Cheese is prepared by curdling milk via the action of a number of enzymes. The substances most commonly used for this purpose in ancient times were **ficin**, obtained as an extract from fig trees, and **rennin**, as rennet, an extract of the lining of the fourth stomach of a multiple-stomach animal, such as a cow.

First scientific report of enzyme discovery was published in 1833, by Anselme Payen and Jean-François Persoz, chemists at a French sugar factory, who reported that alcoholic extract of malt was capable of converting starch into sugar. In fact, the process of removal of husk from the malt, was known as diastasis (a parting or separation). This unknown factor was called **diastase**. It may be noted that the trend of using –ase started much before the term enzyme was coined. Today, diastase refers to any α-, β-, or γ-amylase (all of them are hydrolases) that can break down carbohydrates. In 1834, Schwann discovered that gastric juice can digest the food in test tube and its active principle was named **pepsin.** Until this time catalysis or enzymes were not introduced. It was 1937, when Berzelius proposed the concept of catalysis and gave number of examples from biological systems such as fermentation of sugar by yeast, decomposition of hydrogen peroxide by animal fibrin etc. It's therefore interesting to note that it was biological activity of enzymes that was studied by Berzelius and lead to understanding of catalysis.

Basic idea of catalysis was connected to its cause by Cagniard de Latour in 1838 and later in 1898 by Louis Pasteur, when they proposed that yeast contained active principles called ferments which are the ultimate factors for causing catalysis or fermentation process. In 1850 Louis Pasteur observed that fermentation of sugar into alcohol by yeast is catalyzed by substances, which he referred to as 'ferments' (later named as enzyme). He found that these ferments are always associated with the yeast cells. As he was not successful in dissociating the ferments from the yeast cells, he finally concluded that the ferments are inherent property of yeast cells and are inseparable from them. Similar observations were made by W. F. Kühne in 1876 and coined the word "enzyme" (in Greek), which means, "in yeast" (en = in; zyme = yeast). Leibig (also known as father of biochemistry) was an opponent of Pasteur, who believed that fermentation is an attribute of decay or death while Pasteur believed that it's the process of living cells.

In 1897 another German scientist, Hans Buchner, discovered by accident that fermentation actually does not require the presence of living yeast cells. Buchner made an extract of yeast cells by grinding them and filtering off the remaining cell debris. Then he added a preservative, sugar to the resulting cell-free solution to preserve it for future study. He observed that fermentation, the formation of alcohol from sugar, occurred. Buchner then realized that living cells were not required for carrying out metabolic processes such as fermentation. Instead,

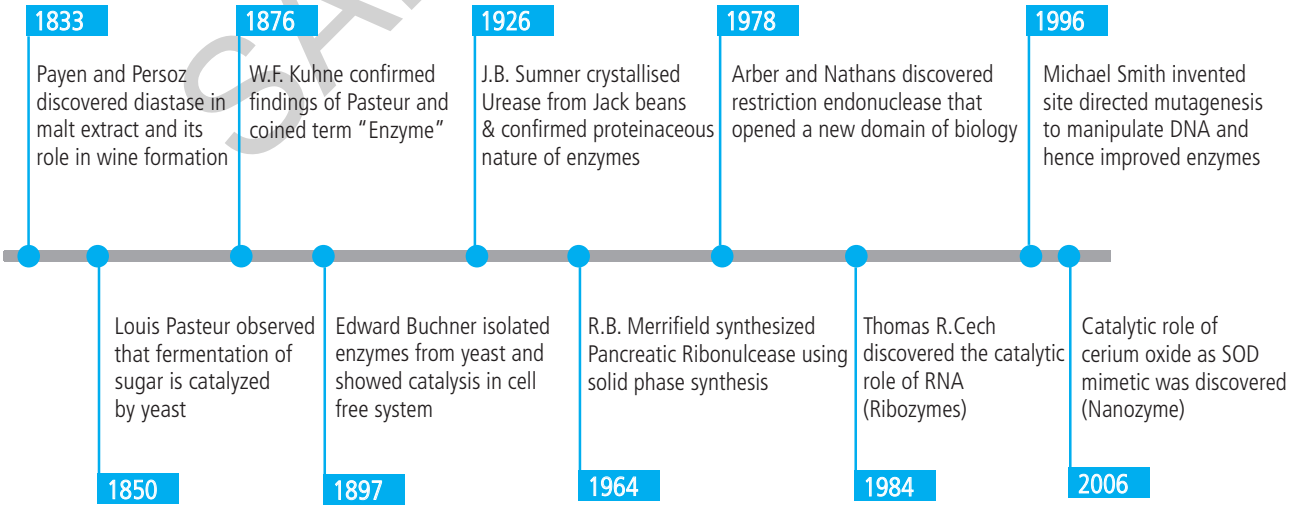


Fig 1.1 Timeline of enzymology

from happening anytime and anywhere. We know that oxidation of paper is a spontaneous reaction, but even if we have oxygen in atmosphere paper does not burn by itself, just because it has an energy barrier which may be crossed by lighting it with a matchstick, that provides activation energy. So, in once sense activation energy is good as it prevent self-destruction of matter and uncontrolled interconversion. Furthermore, as an analogy, an spontaneous reaction can be understood as rolling of a tennis ball kept on a slope (which is spontaneous), but there is a small bulge in the ramp that must be overcome to bring it down (Fig 1.2). This bulge represents activation energy. Activation energy may depend upon nature of reaction, kind of chemical bonds on reactants and even environmental conditions like temperature.

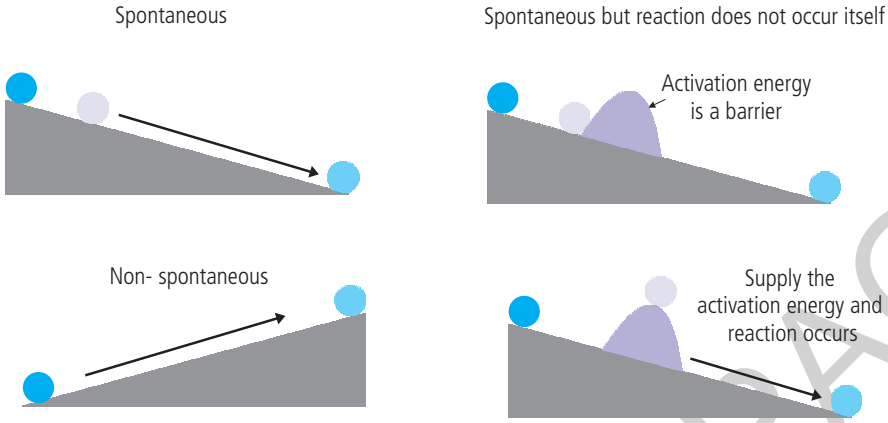


Fig 1.2 Understanding the meaning of activation energy

Now, we may further, imagine another situation, a person has to cross a mountain and reach other side, the amount of time needed to cross the mountain will be more if the mountain is higher. However, if it is cut down and made smaller, the time taken to cross the mountain will be less, hence, the transport across the mountain will be faster. In analogy, decrease in activation energy reduces the uphill barrier and thefore can speed up the reactions (Fig. 1.3).

Caution

It should be noted that different symbols are used for activation energy in various text books, some of the commonly used symbols are E_a, $E^{\ddagger}$ and $\Delta G^{\ddagger}$.

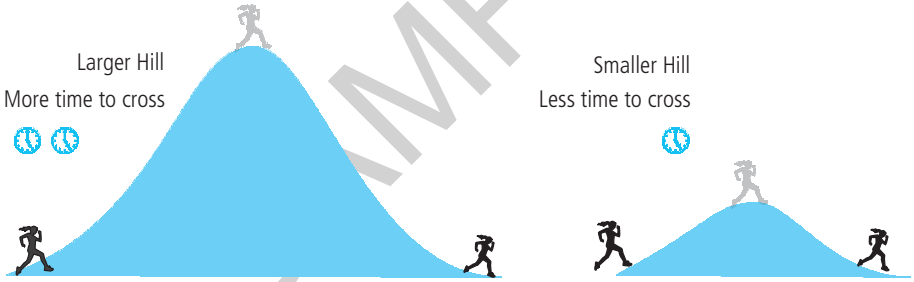
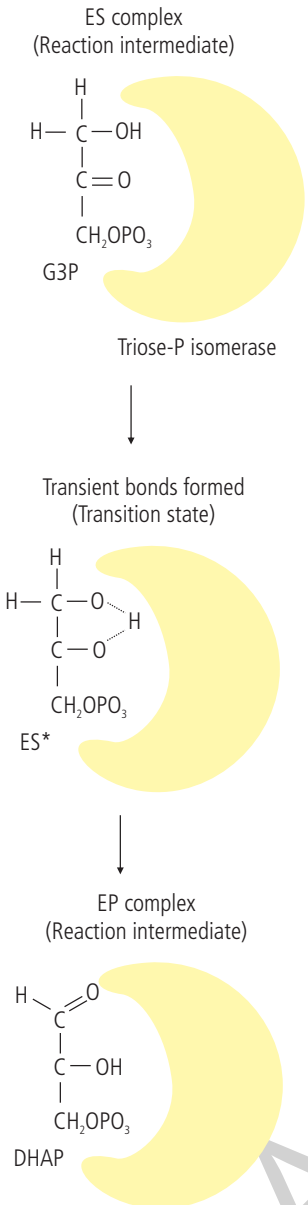


Fig 1.3 Analogy to understand the decrease in activation energy speeds up the process

Although, the example illustrated above was an oversimplified representation of decrease in activation energy and its relationship with rate of reaction, the actual relationship was established by Arrhenius and a kinetic parameter called rate constant was found to be inversely related to the exponent of activation energy. Let us understand this in more detail.

1.3.2 Concept of Transition State

The transition state is the transitory of molecular structure in which the molecule is no longer a substrate but not yet a product. All chemical reactions must go through the transition state to form a product from a substrate molecule. The transition state is the state corresponding to the highest energy along the reaction coordinate. It has more free energy in comparison to the



substrate or product; thus, it is the least stable state. The specific form of the transition state depends on the mechanisms of the particular reaction. It should be noted here that transition state and intermediate state may not have the same meaning. Important difference between the two have been compared in Table 1.2. As an example, we can correlate this with the action of enzyme triose isomerase that interconverts glyceraldehyde-3-phosphate and dihydroxyacetone-3-phosphate. In this reaction there will be a condition, when aldehyde form will be bound to enzymes (intermediate state - ES complex) with intact (-CHO group), another condition will be a temporary state when C_1-O-C_2 linkage will exist, when the aldehyde will be turning into ketone (transition state), while the third state will be the ketone form bound to enzyme just before a release (intermediate state- EP complex).

Table1.2 Differences between intermediate state and transition state

	Intermediate State	Transition State
State	Complexes of reactants	One bond vibration cycle
Stability	Stable	Unstable
Detection	Can be detected and isolated	Cannot be detected and isolated
Gibbs Energy	Low	High
Representation	XX- complex	XX*
Example	ES complex, EP complex,	ES* or EP *

1.3.3 Arrhenius Equation

A physical justification for the increase in rate of reaction by changing temperature was provided by Svante Arrhenius, a Swedish chemist, in 1889. Arrhenius performed experiments that correlated chemical reaction rate constants with temperature. He developed this equation based on his observation that many chemical reaction rates depended on the temperature. Arrhenius equation provides a quantitative relationship between the rates a reaction, temperature and activation energy. The equation can be represented in exponential form as below:

$$k = A \times e^{\frac{-Ea}{RT}}$$

Here, k is the rate coefficient, A is known as pre exponential factor and depends primarily on collisions (more specifically $A = z \times \rho$, where, z is collision factor and ρ is called steric factor), E_a is the activation energy, R is the universal gas constant (8.314×10^{-3} kJ $mol^{-1}K^{-1}$), and T is the temperature (in Kelvin).

As it is apparent from the equation that rate constant is negatively regulated to the exponent of activation energy (E_a), hence, a slight decrease in activation energy results in large increase in k or rate of reaction and vice versa. It is obvious that at higher temperatures, the probability that two molecules will collide is higher and hence the rate of reaction will be higher, due to higher kinetic energy. This higher collision rate also has an effect on the activation energy of the reaction.

The Arrhenius equation can also be represented in logarithmic form as below:

$$\log k = \log A - \frac{E_a}{2.303RT}$$

(2.303 was multiplied to convert $\log_e$ to $\log_{10}$)
Furthermore, an alternate form of this equation at two temperatures can be written as,

$$\log \frac{k_2}{k_1} = \frac{E_a(T_2 - T_1)}{2.303RT_2T_1}$$

Note

k (in lowercase) represents rate constant, while K (uppercase) represents equilibrium constant. Higher the value of k, faster will be the reaction, while higher the value K more will be the amount of product at equillibrium.

$E_a = (8.314)(\ln 1.5)\ 1365\ K - 1373\ K = (8.314)(0.405)0.00274\ K^{-1} - 0.00268\ K^{-1}$
$E_a = (8.314)(\ln 1.5)\ 1365\ K - 1373\ K = (8.314)(0.405)0.00274\ K^{-1} - 0.00268\ K^{-1}$
$= (3.37\ Jmol^{-1}K^{-1})\ 5.87 \times 10^{-5}K^{-1} = 5740\ Jmol^{-1} = 5.73\ kJmol^{-1}$
$= (3.37\ Jmol^{-1}K^{-1})\ 5.87 \times 10^{-5}K^{-1} = 5740\ Jmol^{-1} = 5.73\ kJmol^{-1}$

Comment: This low value seems reasonable because thermal denaturation of proteins primarily involves the disruption of relatively weak hydrogen bonds; no covalent bonds are broken (although disulfide bonds can interfere with this interpretation).

Circe Effect

The utilization of attractive forces to lure a substrate into a site in which it undergoes a transformation of structure. The name was first suggested by an Enzymologist Dr. Willian P. Jenks, inspired by a goddess named "circe" in Greek mythology, who lured Odysseus's men to transform into pigs.

1.4 HOW ENZYMES DECREASE ACTIVATION ENERGY?

Aforesaid illustrations and analogies clearly depict that a decrease in activation energy can cause speeding up of reactions and, as a matter of fact enzymes lower the activation energy and therefore increase the rate of reactions. Larger the decrease, faster will be an enzymatic reaction. But, how does this happen? Why enzymatic reactions require lower activation energy than non-enzymatic reactions? Primarily, every enzyme has a catalytic site, to which substrate binds, and this binding leads to a lesser requirement of energy for making a product. The lowering in activation energy and speeding of reaction by enzymes can be explained in view of three different effects that occur when an enzyme binds with substrate and transform it into product via a transition state.

1.4.1 Entropy Effect

Reaction occur due to collision between the substrates, higher the number of collisions in unit time, faster will be the reactions. However, in absence of enzymes most of the collisions, remain ineffective which consume a lot of energy of reaction without generating product. An effective collision is one where reacting species interact in proper orientation to result in a product molecule. The increased fraction of non-productive collisions is primarily due to higher entropy (randomness) of the molecules. Enzymes, on the other hand precisely place the reactants into proper orientation at their catalytic sites, this reduces the entropy and enhances the number of effective collisions and hence, number of product molecules are formed using same amount of energy. This reduces per molecule consumption of energy to turn them into transition state (Fig. 1.5)

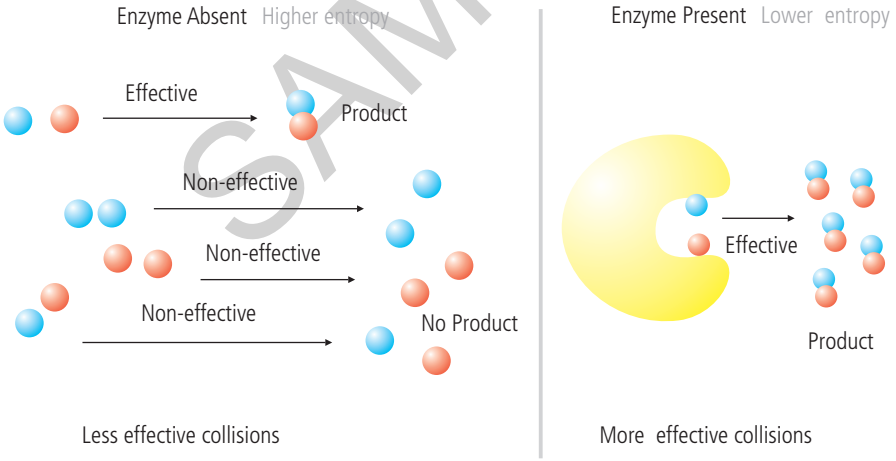


Fig 1.5 Analogy to understand the phenomenon of decrease in activation energy speeds up the process

Effective Molarity

Proximity effect is known to bring more reactants in the close vicinity of active site due to reduced randomness, this increases relative concentration of substrate at active site called effective molarity.

1.4.2 Proximity Effect

Most of the reacting molecules in biological systems are hydrophilic and therefore they are often surrounded by solvent molecules that forms a solvation sphere around reactants. In order

to form a transition state, reactants must remove their solvation sphere and should be closer to each other. Reaction without enzyme expand more energy in bringing the reactants close. However, in enzymatic reactions, the closeness or proximity of atoms is brought about by local microenvironment at the active site of enzyme. The active site has specific set of amino acids, that make the local environment different from surrounding solutions (may be acidic, basic or hydrophobic for different type of enzymes) and therefore leads to the formation of transition state at lower energy cost (Fig 1.6).

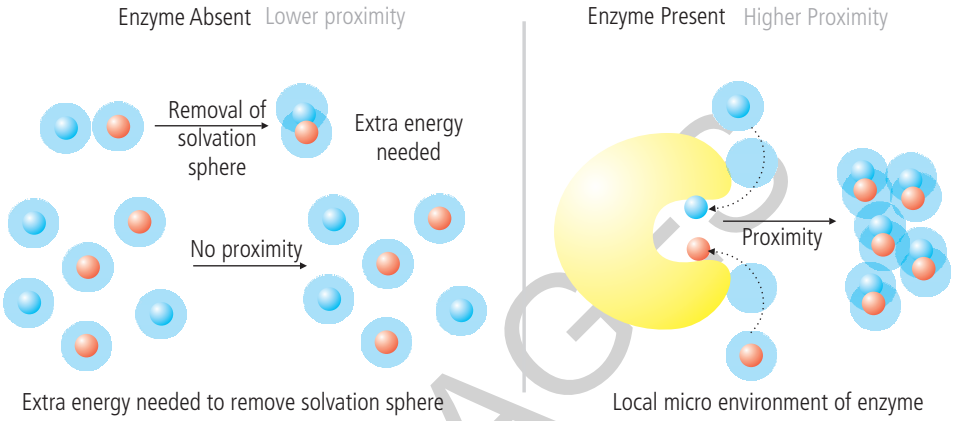


Fig 1.6 Analogy to understand the phenomenon of decrease in activation energy speeds up the process

1.4.3 Strain Effect

Strain Effect

If you push a finger into an inflated baloon, it will constantly impart a reverse force on your hand, trying to regain its original shape. Analogous to this happens during stain effect.

Finally, one more advantage that enzymes confer to form transition state is the strain. As per the induced fit hypothesis, proposed by Koshland, a change in the structure of enzyme is observed during a transition state formation, which is complementary to transition state. Enzyme, in this state imparts a strain on transition state molecules, as it tries to return to native conformation. This strain imparted by enzyme on transition state facilitate the product formation, commonly known as strain effect (Fig 1.7).

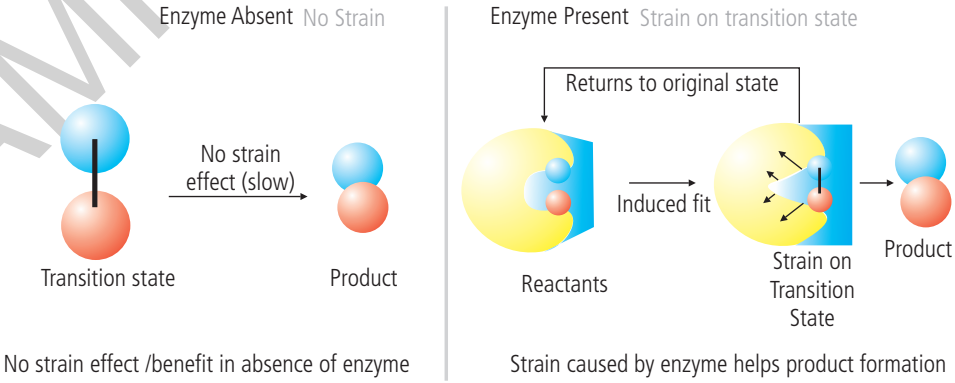


Fig 1.7 Analogy to understand the phenomenon of decrease in activation energy speeds up the process

These three effects, explain the speeding up of reactions by enzymes and lowering in activation energy. Table 1.3 provides a hypothetical view of lesser requirement of activation energy.

Old Yellow Enzyme

One of the trivial name of enzyme was old yellow enzyme, which is today known as NADPH dehydrogenase (EC 1.6.99.1). Other names in common use include $NADPH_2$ diaphorase, NADPH diaphorase, OYE, diaphorase, dihydronicotinamide adenine dinucleotide phosphate dehydrogenase, NADPH-dehydrogenase and NADPH-diaphorase.

Seventh Class

Earlier, there were only six classes of enzymes, A new class was added named tranlocase in August 2018. This includes the enzymes catalysing the translocation of molecules or ions across the plasma membrane.

Table 2.1 Common databases for retrieval of information on enzymes

S. No.	Name	Database for	URL / Website
1	IUBMB^	Database for	http://www.sbcs.qmul.ac.uk/iubmb/enzyme/
2	Explore Enz	Enzyme Names and EC. No.	https://www.enzyme-database.org/index.php
3	Enzyme	Enzyme Nomenclature	https://www.ncbi.nlm.nih.gov/Class
4	Expasy – Enzyme	Enzyme Nomenclature	https://enzyme.expasy.org/
5	BRENDA	Enzyme Nomenclature	http://www.brenda-enzymes.org/
6	MACiE*	Comprehensive enzyme info.	https://www.ebi.ac.uk/thornton-srv/
7	SABIO-RK	Enzyme mechanism	http://sabio.h-its.org/
8	EzCatDB	Enzyme reaction kinetics	http://ezcatdb.cbrc.jp/EzCatDB/
9	GTD	Enzyme catalytic mechanisms	https://randr.nist.gov/enzyme/
10	MetaCyc	Enzyme thermodynamics info.	http://metacyc.org/
11	KEGG	Metabolic pathways (Enzymes)	http://www.genome.ad.jp/kegg/
12	PDB	Metabolic pathways (Enzymes)	http://www.rcsb.org/pdb/

^International Union of Biochemistry and Molecular Biology, *Mechanism, Annotation and Classification in Enzymes.

2.2.1 Principles of Enzyme Commission Recommendations

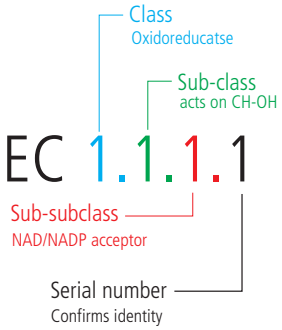
The recommendations of enzyme commission can be summarized in the form of three basic principles that forms the basis of actual classification and nomenclature schemes of enzymes.

The first general principle states that names of enzymes, especially those ending in -ase, should be used only for single enzymes, i.e. single catalytic entities. They should not be applied to systems containing more than one enzyme. When it is desired to name such a system on the basis of the overall reaction catalyzed by it, the word system should be included in the name. For example, the system catalyzing the oxidation of succinate by molecular oxygen, consisting of succinate dehydrogenase, cytochrome oxidase, and several intermediate carriers, should not be named succinate oxidase, but it may be called the succinate oxidase system.

The second general principle is that enzymes are principally classified and named according to the reaction they catalyse. The chemical reaction catalyzed is the specific property that distinguishes one enzyme from another, and it is logical to use it as the basis for the classification and naming of enzymes. Therefore, enzymes from different sources such as bacterial, plant or animal species are classified as one entry. Similarly, isozymes are assigned same name.

A third general principle adapted is that the enzymes are divided into groups on the basis of the type of reaction catalyzed, and this, together with the name(s) of the substrate(s) provides a basis for naming individual enzymes. It is also the basis for classification and code numbers.

2.2.2 Enzyme Commission Number



Each enzyme is given a unique four-digit code, known as the Enzyme Commission, or EC, Each of the seven main classes is further subdivided. Each digit is separated by a period or dot. First digit represent class. As there are seven classes of enzymes, the first digit will be from 1–7. The second digit represents, sub-class. The subclass generally contains information about the type of compound or group involved, for each class the definition of subclass mat be different. The third digit represents sub-subclass, which further elucidates the differences in individual reactions. The fourth digit is a serial number that is used to identify the individual enzymes.

Examples 1.1.1.1 represents an enzyme belongs to class 1 i.e. oxidoreductase, subclass 1, which means, it acts on the CH–OH group of donors whereas sub-sub class 1 means it

utilized, NAD or NADP is the acceptor, and serial no. 1 which confirms its identity as alcohol dehydrogenase.

Partial EC numbers are incomplete EC number with uncertain serial number. Partial EC numbers look like EC numbers except the last number is replaced by a dash, e.g. 2.1.1.- . Partial EC numbers should not be used for functional assignment. Partial EC numbers are used for two primary reasons one, partial knowledge (2.1.1.- is the general class of methyltransferases) or second if the updated EC no. has not received an EC number yet eg. EC 2.3.4.n. Sometimes sub-classes may have values 99, which represents a group of enzymes, for which a specific category has not been elucidated or they do not fit into existing categories, and therefore a category 'others' has been assigned. Eg. EC 4.99 represents other lyases and EC 5.99 represents other isomerases.

TC Number

Similar to EC numbers or enzyme commission numbers used for the identification of enzymes, TC number or transport commission numbers are used for the identification of membrane transporters.

2.2.3 Common Names and Systematic Names

The first enzyme commission gave much thought to the question of a systematic and logical nomenclature for enzymes, and finally recommended that there should be two nomenclatures for enzymes, one systematic, and one working or trivial. The **systematic name** of an enzyme, formed in accordance with definite rules, showed the action of an enzyme as exactly as possible, thus identifying the enzyme precisely (Substrates are listed first (colon separated) followed by the type of catalytic reaction with the suffix -ase). The **trivial name (common name)** was sufficiently short for general use, but not necessarily very systematic; in many cases it was a name already in current use. Table 2.2 enlists some of the common names and systematic names of some enzymes.

Systematic Names

Systematic names consist of two parts. The first contains the name of the substrate or, in the case of a bimolecular reaction, of the two substrates separated by a colon. The second part, ending in -ase, indicates the nature of the reaction.

The introduction of (often cumbersome) systematic names was strongly criticized. In many cases the reaction catalyzed is not much longer than the systematic name and can serve just as well for identification, especially in conjunction with the code number. The commission for revision of enzyme nomenclature discussed this problem at length, and a change was made. It was decided to give the trivial names more prominence in the enzyme list, i.e. they now follow immediately after the code number, and are described as common name. Also, in the index the common names are indicated by an asterisk. Nevertheless, it was decided to retain the systematic names as the basis for classification for the following reasons:

Accepted Names

The most commonly used name for the enzyme, provided that it is neither unambiguous nor misleading. Most of the accepted names are common names (or trivial names) followed by EC numbers.

- The code number alone is only useful for identification of an enzyme when a copy of the enzyme list is at hand, whereas the systematic name is self-explanatory;
- The systematic name stresses the type of reaction, the reaction equation does not;
- Systematic names can be formed for new enzymes by the discoverer, by application of the rules, but code numbers should not be assigned by individuals;
- Common names for new enzymes are frequently formed as a condensed version of the systematic name; therefore, the systematic names are helpful in finding common names that are in accordance with the general pattern.

Recomended Names

Each enzyme is assigned a recommended name; usually at the suggestion of the person who submits the details. The rules defined by IUBMB are used to classify enzymes, once approved it becomes accepted name.

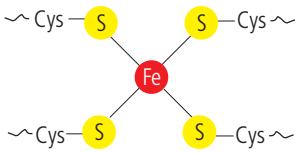
Table 2.2 Examples of recommended names (common names or trivial names) and systematic names

S. No.	Common Names	Systematic Names	Reaction
1	Alcohol dehydrogenase	Alcohol:NAD^+ oxidoreductase	Oxidation of Alcohols
2	DNA polymerase	dNTP:DNA dNMPtransferase	Polymerization of nucleotides
3	Methyltransferase	Donor:Acceptor methyltransferase	Transfer of methyl group
4	Urease	Urea:Amidohydrolase	Hydrolysis of urea
5	Hexokinase	ATP:D-hexose 6-phosphotransferase	Phosphorylation of hexose
6	Pyruvate Kinase	ATP:pyruvate 2-O-phosphotransferase	Formation of pyruvate from PEP
7	Cytochrome oxidase	Ubiquinol:oxygen oxidoreductase	Oxidative phosphorylation

It also occurs as a component of iron-sulfur clusters in enzymes that are involved in one-electron transfer processes such as NADH dehydrogenase and succinate dehydrogenase. Cobalt containing enzymes have cobalt atom bound within a **corrin ring.** Spatial geometry is also a concern, and therefore metal ions must adhere to strict geometric configurations around the metal-binding site. Iron (Fe^{2+}) and copper (Cu^{2+}) is present in many hydrolase enzymes which utilize multivalent oxidation states for reaction with oxygen. Iron is usually present in two different form, one is inorganic components as iron-sulphur clusters, or conjugated to organic components as heme. Iron in the form of heme may therefore be considered as coenzyme and shall be discussed under the section organic cofactor.

Nickel (Ni^{2+}) is rarely found as a component of metalloenzymes, but urease from jack bean is an exception. The occurrence of manganese (Mn^{2+}) and calcium (Ca^{2+}) in metalloenzymes is also somewhat rare. By contrast, Zinc (Zn^{2+}) is an important and widely utilized metal for electrophilic catalysis. Copper zinc superoxide dismutase is a metalloenzyme that uses copper and zinc to help catalyze the conversion of superoxide anion to molecular oxygen and hydrogen peroxide. Thermolysin is a protease that uses a tightly bound zinc ion to activate a water atom, which then attacks a peptide bond. Aconitase is one of the enzymes of the citric acid cycle; it contains several iron atoms bound in the form of iron-sulfur clusters, which participate directly in the isomerization of citrate to isocitrate. Other metal ions found as cofactor in metalloenzymes include molybdenum (in nitrate reductase), selenium (in glutathione peroxidase), nickel (in urease), and vanadium (in fungal chloroperoxidase).

A typical iron-sulphur cluster



[1Fe -0 S type]

Note

Not all enzymes that catalyze a particular reaction have the same requirement for a metal. Thus, fructose bisphosphate aldolase from yeast and bacteria utilize Zn ions, whereas the same enzyme from muscle uses a Schiff Base intermediate to activate the substrate

3.2.2 Loosely Associated Metal Ions

Alkaline earth metals magnesium ions (Mg^{2+}) and manganese ions (Mn^{2+}) are found most commonly in biological systems, especially in loosely bound state in metal-activated enzymes. The largest group of metal-activated enzymes contains the phosphotransferases that catalyze the transfer of the terminal phosphoryl group of ATP to an acceptor molecule that can be an alcohol, carboxylic acid, nitrogenous compound, or a phosphorylated compound. The metal may activate the substrate, engage the enzyme directly, or enter into equilibrium with the enzyme exploiting its ionic charge to render a more favorable substrate binding or catalytic environment. Therefore, metal-activated enzymes require the metal to be present in excess, almost 2-10 times more than the enzyme concentration. Because the metal cannot be bound in a more permanent way, metal-activated enzymes typically lose activity during purification. Pyruvate kinase also differs from most other phosphotransferases in its requirement for K and its inhibition, rather than activation, by calcium ions. Some of the metal containing and metal requiring enzymes are enlisted in Table 3.2

Practice Question

Question: Which of the following enzymes or proteins do not contain iron?

Aconitase, Cytochrome C, DNA primase, Lipoic acid synthase, Homoaconitase, Calmodulin, Calrecticulin, Myoglobin, Hemoglobin.

Hint: Calcium containing enzymes do not require iron, i.e Calmodulin and Calrecticulin

Metallozymes

Metallozymes or metalloenzymes represent the set of enzymes which have metal or their ions as integral part of their structure. These enzymes represent an evolutionary event where catalytic abilities of metal ions would have been coupled to proteins in early life forms. Not all metal ions in metalllozymes are catalytic, some may have non-catalytic role as well.

3.2.3 Modes of Interaction of Metal Ions with Enzyme

Interaction of metal ions with the enzymes can be studied using Nuclear Magnetic resonance (NMR), Electron Spin Resonance (ESR) and Proton Relaxation Rate (PRR). Albert Mildvan in 1960, described that the interaction of metal ions with that of the enzyme, can occur in different ways and hence different type of ternary complexes may be formed. Fig 3.2 illustrates some of the common type of metal complexes with enzyme and substrates.

Note

Metal ions in metallozymes are often linked via his, asp, or cys through coordinate bonds.

Table 3.2 Examples of various enzymes that utilize metal ions as cofactor

Metal ion	Enzyme
Metal Containing Enzymes (Metalloenzymes)	
Ca^{2+}	Amylase, Galactosyltransferase, Thermolysin
Fe^{2+}	Catalase, NADH:dehydrogenase, Nitrogenase, Peroxidase, Succinate dehydrogenase, Xanthine oxidase
Co^{2+}	Dioldehydrase, Glycerol dehydratase, Methylmalonyl-CoAmutase, Ribonucleotide reductase
Cu^{2+}	Cytochrome c oxidase, dopamine-b-hydroxylase, Superoxide dismutase
Mn^{2+}	Arginase, Histidine-ammonia lyase, Pyruvate carboxylase
Zn^{2+}	Alcohol dehydrogenase, Carbonic anhydrase, carboxypeptidase, superoxide dismutase, thermolysin
Mo^{2+}	Nitrogenase, Xanthine oxidase, nitrate reductase, sulfite oxidase
Ni^{2+}	Urease, Ni-Fe hydrogenase
Se	Glutathione peroxidase
V	Fungal chloroperoxidase
Metal Requiring Enzymes (Metal-activated enzymes)	
K^{+}	Pyruvate Kinase
Mg^{2+}	DNase, RNase, ATPase, Glucose-6-phosphatase, Hexokinase

Enzyme Bridge Complexes: The enzyme is sandwiched between metal ion and substrate, i.e. M-E-S complex is formed. E.g. Carboxypeptidase enzyme, which forms the complex with zinc ions (Zn^{2+}) ions bridged between enzyme and substrate.

Substrate Bridge Complexes: The substrate is sandwiched between enzyme and metal ion, i.e. E-S-M complex is formed. E.g. creatine kinase, which forms a complex, creatine-E-ATP-Mg^{2+}

Metal Bridge Complexes: The metal ion is sandwiched between enzyme and substrate, therefore known as metal bridge complex. E.g. E-M-S complex is formed. e.g. Triose Isomerase (TIM), which forms, - Enzyme Mn^{2+}- PEP complex.

Cyclic Metal Bridge Complexes: The metal ion is sandwiched between enzyme and substrate and also connected in cyclic manner with substrate i.e. E-M-S complex is formed. e.g. Pyruvate kinase, Enolase, Glutamate ammonia ligase, which forms, Enzyme-metal ion- Substrate complex. Pyruvate kinase forms a complex with metal ion (Zn^{2+}) is linked to substrate as well as metal ion and substrate also linked to enzyme (Fig 3.2).

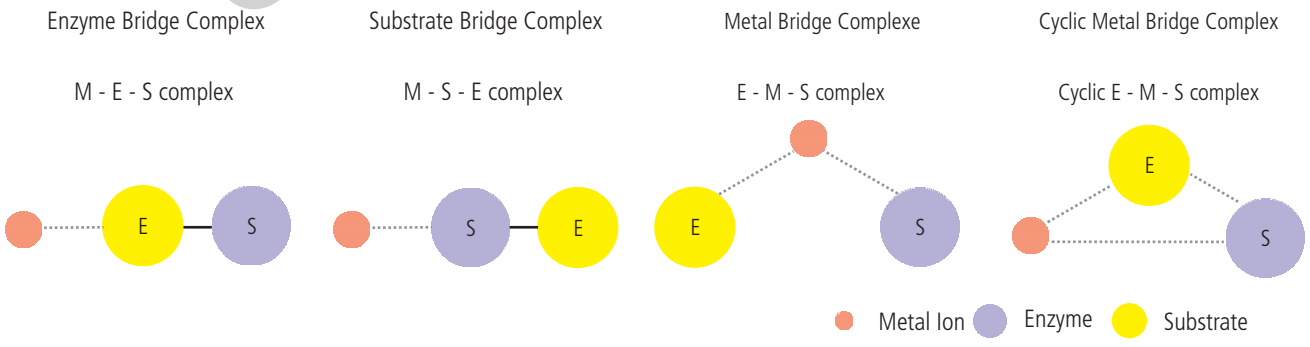


Fig 3.2 Outline of various type of metal ion interactions with enzymes

known as retinoic acid receptors (RARs) which are bound to DNA as heterodimers with retinoid "X" receptors (RXRs). Nuclear receptors in the gonads increase gene expression and maintain reproductive tissues while nuclear receptors in epithelial cells regulate cell differentiation. The biochemistry of vision can be understood as following sequence of events.

Biochemistry of vision: The role of vitamin A in the visual cycle is specifically related to the **retinal form**. Within the visual sensory cells of eye, 11-cis-retinal is bound to the protein called opsin and forms rhodopsin (in rod cells) or iodopsin (in cone cells) at conserved lysine residues. As light enters the eye, the 11-cis-retinal is isomerized to the all-trans form. The all-trans retinal (all double bonds in trans isomeric form) retinal dissociates from the opsin in a series of steps called photo-bleaching. This isomerization induces a nervous signal along the optic nerve to the visual center of the brain. After separating from opsin, the all-trans- is recycled and converted back to the 11-cis-retinal form by a series of enzymatic reactions. In addition, some of the all-trans retinal may be converted to all-trans retinol form and then transported with an inter-photoreceptor retinol-binding protein (IRBP) to the pigment epithelial cells. Further esterification into all-trans retinyl esters allow for storage of all-trans-retinol within the pigment epithelial cells to be reused when needed. The final stage is conversion of 11-cis-retinal rebinds to opsin that reforms rhodopsin (visual purple) in the retina. Rhodopsin is needed to see in low light (contrast) as well as for night vision. Kühne showed that rhodopsin in the retina is only regenerated when the retina is attached to retinal pigmented epithelium, which provides retinal. It is for this reason that a deficiency in vitamin A will inhibit the reformation of rhodopsin and lead to one of the first symptoms, night blindness.

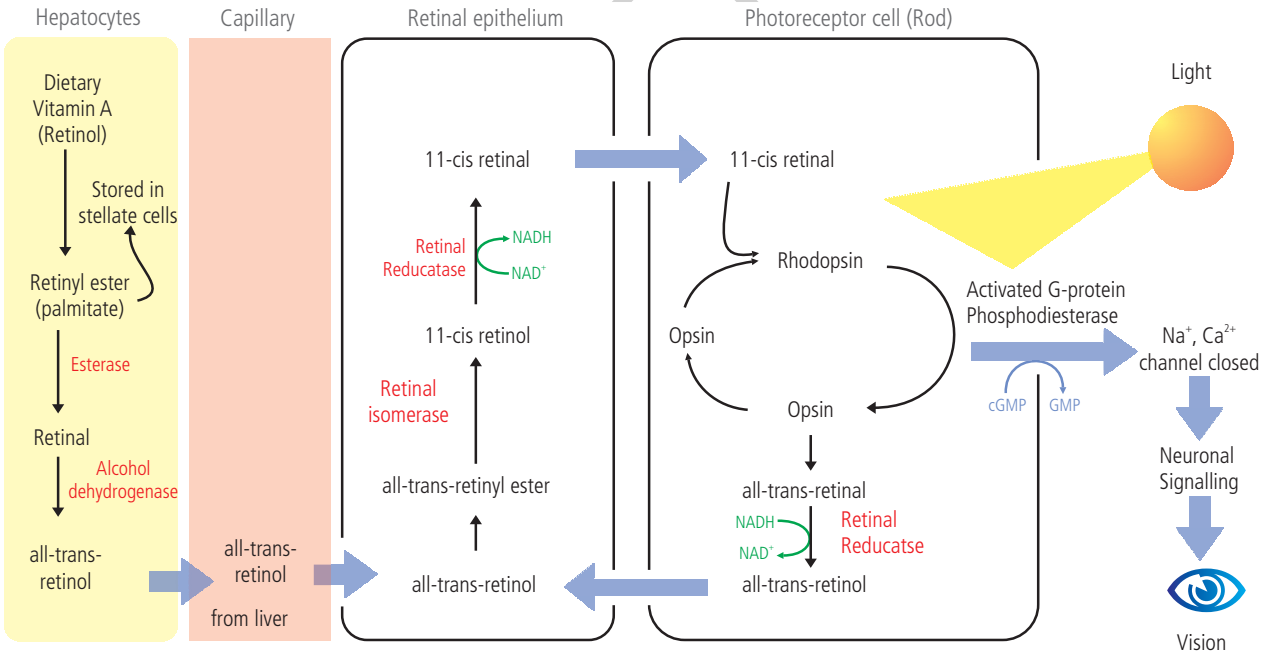


Fig 3.15 Biochemistry of vision

3.5.2 Vitamin B

Vitamin B is biochemically more important vitamin due to its involvement in various metabolic reactions, as common cofactor of enzymes, different vitamers have different functions, which have been described briefly as follows:

Shortcomings: The graph of Michaelis-Menten equation in its original form (hyperbolic) has a shortcoming, that V_{max} and K_m cannot be determined precisely. As one has to consider taking infinite substrate concentration, in order to determine precise V_{max}, which is mere approximation.

> **Equation of Line**
>
> As per coordinate geometry, every curve has an equation, so is a line. A general equation $y = mx + c$ represents two variables x and y plotted on two axes respectively. c is called intercept on y axis (point where line touches y-axis) and m is called slope (incline towards y axis). Higher slope, means more vertical line (recall as it is difficult to climb on higher slope).

4.4.3 Lineweaver Burk Plots

In order to rectify the shortcomings of hyperbolic curve, Hans Lineweaver and Dean Burk in 1934, developed a simple method by inverting the existing Michaelis-Menten equation, which fits well into the equation of a straight line. Fig 4.6 represents typical lineweaver-burk plot and various components on the plots. Following is the mathematical conversion of Michaelis-Menten equation into lineweaver burk equation.

Original MM equation

$$v_0 = \frac{V_{max}[S_0]}{K_m + [S_0]}$$

On inverting both the sides we get,

$$\frac{1}{v_o} = \frac{K_m + [S_0]}{V_{max}[S_0]} \quad \text{or} \quad \frac{1}{v_o} = \frac{K_m}{V_{max}[S_0]} + \frac{[S_0]}{V_{max}[S_0]} \quad \text{or} \quad \frac{1}{v_o} = \frac{K_m}{V_{max}[S_0]} + \frac{1}{V_{max}}$$

This equation, can now be compared with equation of line $y = mx + c$,

$$\text{Where, } y = \frac{1}{v_o},\ x = \frac{1}{[S_0]},\ m = \frac{K_m}{V_{max}} \text{ and } c = \frac{1}{V_{max}}$$

Now, if we plot inverse of rate of reaction (v_0) on y axis and inverse of substrate concentration [S_0] on x axis, a straight line is obtained. It must be noted that point at which the line intersects y axis (c) represents $\frac{1}{V_{max}}$ and the point at which the same line intersects x axis represents -1/K_m. the slope of this line represents $\frac{K_m}{V_{max}}$

Hint: In order to determine the value of point where line intersects at x-axis, place the value of y as zero (as value of y is zero at that point) and then determine the value of x by solving the equation.

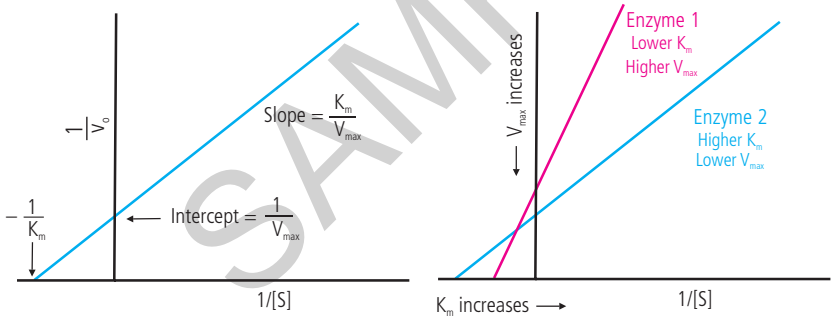


Fig 4.6 Double reciprocal plot or Lineweaver Burk plot. Note that on if the line intersects closer to origin, values of V_{max} and K_m are higher than the line intersecting farther from origin.

It must also be noted here that, as the line intersects, farther from origin on y axis, value of $1/V_{max}$ increases or V_{max} decreases. The lineweaver burk plot remains the most preferred plot for enzymologists. However, enzyme kineticists prefer modified versions as described below.

Shortcomings: The criticism of the lineweaver Burk plot was mainly about the extrapolation of $1/v_0$ to determine the value of K_m. Additionally, undue weightage was given to the measurements at low substrate concentrations.

Original Double reciprocal equation

$$\frac{1}{v_o} = \frac{K_m + [S_0]}{V_{max}[S_0]}$$

Multiplied on both sides by $[S_0]$, we get,

$$\frac{1}{v_o} \times [S_0] = \frac{K_m}{V_{max}[S_0]} \times [S_0] + \frac{[S_0]}{V_{max}[S_0]} \times [S_0]$$

On rearranging we get,

$$\frac{[S_0]}{v_o} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \times [S_0]$$

This equation, can now be compared with equation of line $y = mx + c$,

$$\text{Where, } y = \frac{[S_0]}{v_o},\ x = [S_0],\ m = \frac{1}{V_{max}} \text{ and } c = \frac{K_m}{V_{max}}$$

Now, if we plot the graph between rate of reaction $[S_0]/ v_o$ on y axis and $[S_0]$ on x axis, a straight line is obtained. It must be noted that point at which the line intersects y axis (c) represents $\frac{K_m}{V_{max}}$ and the slope of the line represents $\frac{1}{V_{max}}$. So, there is no absolute requirement of extrapolation to determine the value of K_m using this equation. Also, the point at which the same line intersects x axis represents $-K_m$.

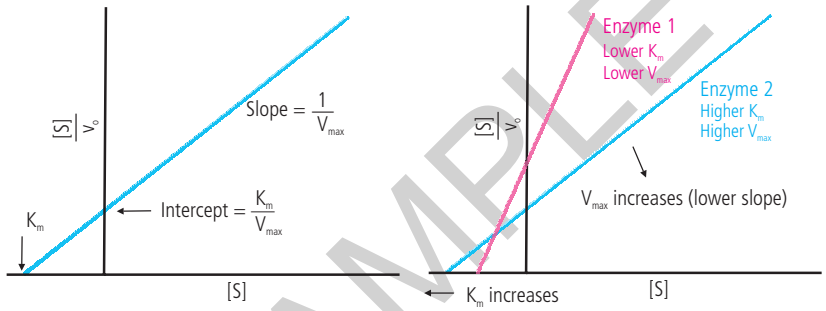


Fig 4.8 Hanes woolf Plot

4.4.6 Eisenthal - Cornish - Bowden plot

One more approach, which was quite unusual and different, was developed by Robert Eisenthal and Cornish-Bowden in 1974. This was an unusual approach because the graph that was proposed, was between two constants V_{max} and K_m, which is usually not observed in standard mathematics text. However, the equation holds good mathematically,

On rearranging double reciprocal equation as follows:

$$\frac{1}{v_o} = \frac{K_m + [S_0]}{V_{max}[S_0]}$$, multiplying both sides by V_{max}, we get,

$$\frac{V_{max}}{v_o} = \frac{K_m + [S_0]}{[S_0]} = \frac{K_m}{[S_0]} + 1$$

Quick summary

Feature	MM	LB	EH	HW
Y-axis	v_0	$\frac{1}{v_0}$	v_0	$\frac{[S]}{v_0}$
X-axis	[S]	$\frac{1}{[S]}$	$\frac{v_0}{[S]}$	[S]
Y-intercept	-	$\frac{1}{v_{max}}$	V_{max}	$\frac{K_m}{V_{max}}$
X-intercept	-	$\frac{-1}{v_{max}}$	$\frac{v_{max}}{K_m}$	$-K_m$
Slope	-	$\frac{K_m}{v_{max}}$	$-K_m$	$\frac{1}{V_{max}}$

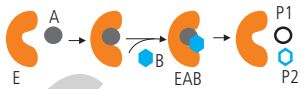
MM – Michaelis-Menten; LB – Lineweaver Burk; EH – Eadie Hofstee; HW – Hanes Woolf equation

(the substrates may bind in a random sequence or in a specific order), called **ternary complex mechanism**. In Second case, the first substrate is converted to product and dissociates before the second substrate binds, so no ternary complex is formed, this type of kinetics is known as **Ping-Pong, or double-displacement**, mechanism. Steady-state kinetics and their respective Lineweaver Burk plots can often help distinguish among these possibilities.

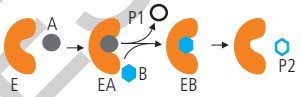
5.2.1 Ternary Complex Kinetics

Ternary complex formation means the two substrates remain bound to enzyme at any point of time in the reaction leading to the formation of a complex that has three components (hence, ternary). However, the formation of ternary complex has been proposed to occur in two different ways. First, the substrate A always binds first followed by binding of substrate B, called sequential order. Second, any of the two substrates can bind with enzymes and then next substrate may bind to form ternary complex, called random order, as represented below:

Ternary complex kinetics



Ping- Pong kinetics



Sequential order: $A + E \rightarrow AE + B \rightarrow AEB \rightarrow E + P1 + P2$

Random order: $A + E \rightarrow AE + B \rightarrow AEB \rightarrow E + P1 + P2$ or $B + E \rightarrow BE + A \rightarrow AEB \rightarrow E + P1 + P2$

Here A and B are two substrates for the reaction, E is enzyme and P1, P2 are two different products.

Examples of enzymes with ternary-complex mechanisms include reactions catalyzed by glutathione S-Transferase, dihydrofolate reductase, and DNA polymerase.

5.2.2 Ping-Pong Kinetics

The ping pong kinetics does not involve formation of a ternary complex, instead, the products are released sequentially one by one from each of the substrates as shown below:

$$A + E \rightarrow AE + P1 \rightarrow EB \rightarrow E + P2$$

Here A and B are two substrates for the reaction, E is enzyme and P1, P2 are two different products.

Examples of enzymes with ping–pong mechanisms include some reactions catalyzed by oxidoreductases such as thioredoxin peroxidase, transferases such as acylneuraminate cytidylyl transferase and serine proteases such as trypsin and chymotrypsin.

5.2.3 Differences in the Kinetics Pattern on Lineweaver Burk Plots

Steady-state kinetic analysis of bisubstrate reactions can be performed by using standard kinetic methods that involve determination of velocity of enzymatic reactions at different substrate concentration. Usually, concentration of one of the substrate is kept constant and that of the second is varied, thereby producing several lines on Lineweaver Burk plot, each line represents variation of velocity at different concentrations of one of the substrate, keeping the concentration of other substrate constant. The kinetic pattern of two different mechanisms followed by enzymes is reflected on the graphs which can be used to assess the difference. Enzymes following ternary complex mechanism produces intersecting lines on the graph while enzymes following ping pong mechanism produces parallel lines on a double reciprocal plot (Fig 5.1).

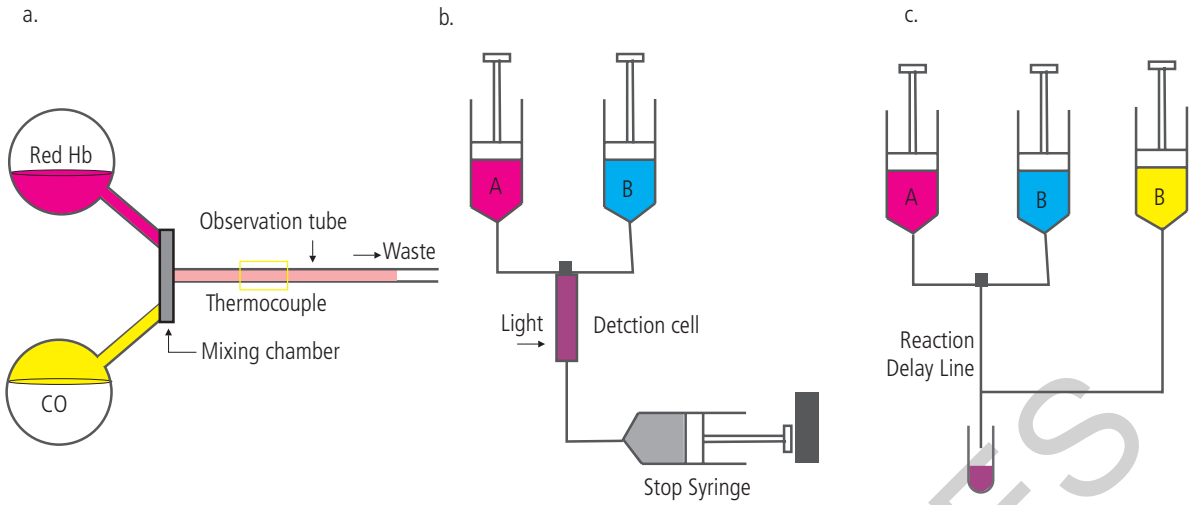


Fig 5.3 Apparatus of three different type of flow methods of studying pre-steady state enzyme kinetics a. Continous flow apparatus b. Stopped flow apparatus c. Rapid quenching apparatus

Table 5.2 Comparison of methods to study pre-steady state kinetics (continuous flow method is not in use)

Parameter	Stopped Flow method	Rapid Quenching Method	Relaxation Method
Nature of method	Mixing	Mixing	Temperature Jump
Reactions Applicability	Reversible and Irreversible	Reversible and Irreversible	Reversible reactions only
Time range	Microseconds to Minutes	Microseconds to Milliseconds	Picoseconds to nanoseconds
Reaction curve (with time)	Continous	Discrete	Continous

extremely rapid pressure or temperature (such as 5°C change in few microseconds), and as a result the position of equilibrium changes slightly, then the system will relax towards the new equilibrium position according to the relationship

$$\Delta[A] = \Delta[A]_0^{-t/\tau}$$

Where [A] is the difference between the concentration of A at time t and the new equilibrium position, i.e.

$\Delta[A] = [A] - \Delta[A]_{eq}$ and $[A]_0$ is the difference between concentration of A immediately after the temperature or pressure change at the new equilibrium position. τ is the relaxation time. The same equation can also be transformed and represented in logarithmic form as

$$\ln[\Delta A] = \ln[\Delta A]_0 - t/\tau$$

This also gives a linear equation, which can be plotted on graph. A graph of ln [ΔA] on y-axis and t on x-axis shall give a straight line, with slope of the line representing $-1/\tau$.

This relation holds true for all chemical reactions where the perturbation in equilibrium is small, but the meaning of τ in terms of reaction constants depends upon the actual reaction mechanism. The meaning of τ for most of the complex reactions is very complicated and can be identified using individual steps and particular rate constant.

5.4 KINETICS OF LIGAND- RECEPTOR BINDING

Besides enzymes, the biding between a protein receptor (a macromolecule) and a ligand (small molecules) is often observed in many other instances. Antigen- antibody interactions, membrane

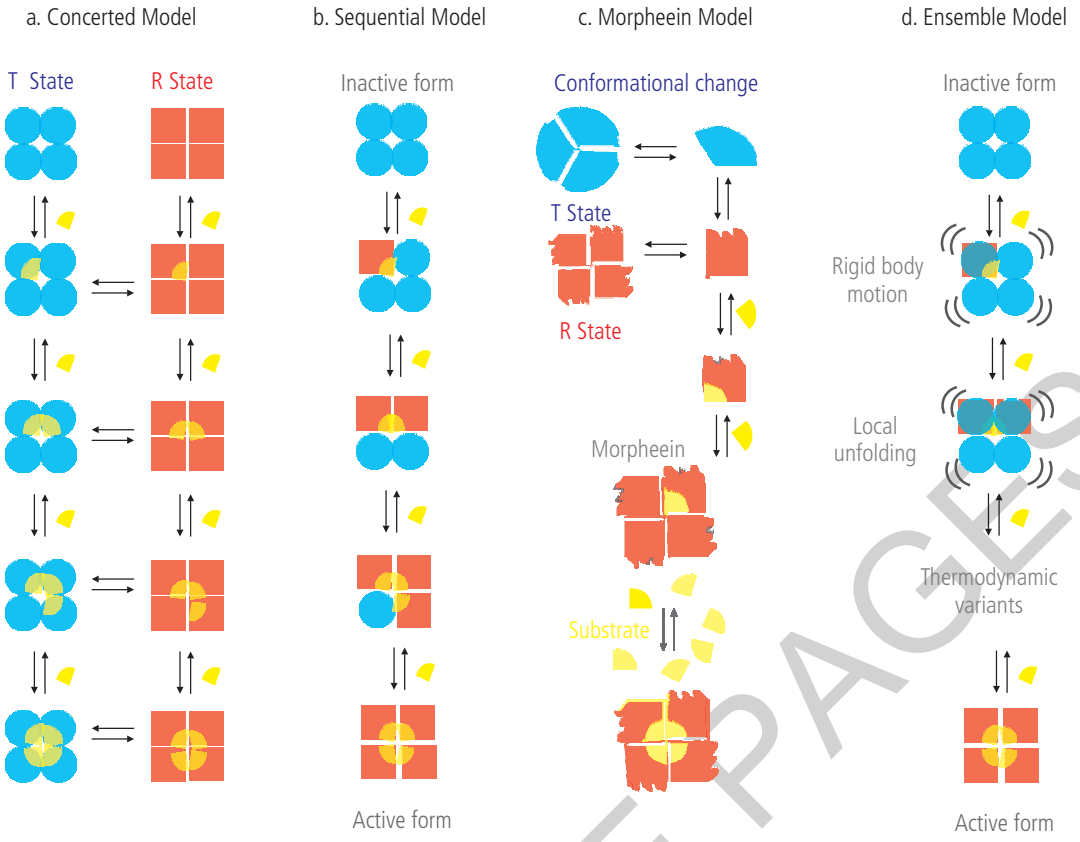


Fig 5.11 Four different models of Allosteric cooperativity a. Sequential Model, b. Concerted Model and c. Morpheein Model, d. Ensemble Model (approximated)

Therefore, at the given ratio of V_{max} i.e. 0.8 and 0.1, we can obtain cooperativity index as follows,

When $v = 0.8\ V_{max}$, then $0.8 = \frac{[S]^n_{0.8}}{K' + [S]^n_{0.8}}$

Or $[S]_{0.8} = \sqrt[n]{4K'}$

Similarly, When $v = 0.1\ V_{max}$, then $0.1 = \frac{[S]^n_{0.1}}{K' + [S]^n_{0.1}}$, or $[S]_{0.1} = \sqrt[n]{K'/9}$

Hence, ratio of substrate concentrations at two velocities (cooperativity index) $= \frac{[S]_{0.8}}{[S]_{0.1}} = \frac{\sqrt[n]{4K'}}{\sqrt[n]{K'/9}} = \sqrt[n]{36}$

Similarly for a ratio of $\frac{[S]_{0.9}}{[S]_{0.1}} = \sqrt[n]{81}$

Practice Question

Q.uestion: The $\frac{[S]_{0.9}}{[S]_{0.1}}$ ratio for an enzyme that obeys sigmoidal kinetics was found to be 5.5, what is the apparent value of n (n_{app})

Solution: As calculated above $\frac{[S]_{0.9}}{[S]_{0.1}} = \sqrt[n]{81}$, we can solve further using log

$$n = \frac{\log 81}{\log 5.5} = \frac{1.91}{0.74} = 2.58$$

Table 6.1 Summary of various patterns of feedback regulation

S. No.	Patten	Mechanism	Example
1	Cooperative (Synergistic)	End product of each branch of pathway inhibits the enzyme at the first step of the pathway	Phosphoribosylamine synthase
2	Concerted (Multivalent)	End of product of each branch of the pathway does not inhibit first enzyme, but together they inhibit	Beta- aspartyl kinase
3	Partial cumulative	End of product of each branch of the pathway has slight inhibition of first enzyme, but together they inhibit more. (like x inhibits 20% Y inhibits 10% , together >30%)	Glutamine synthetase
4	Additive	End of product of each branch of the pathway has some inhibition of first enzyme, and together they inhibit equal to sum of two inhibition effects.	Beta- aspartyl kinase
5	Sequential	End product of each branch of pathway inhibits the first enzyme of the branching point and not the first enzyme of entire pathway.	DHAP synthase

regulating different enzymes. Based on this fact, feedback regulation can follow several patterns such as cooperative regulation (synergistic), concerted regulation (multivalent), Partial cumulative regulation, additive regulation and sequential regulation. Table 6.1 summarizes the different type of feedback regulations with specific examples of enzymes.

6.2.5 Reciprocal Regulation of Enzymes

Among the various patterns of regulation and relay of regulatory signals, another important mechanism is known as reciprocal regulation. This mechanism exists generally to avoid futile cycles. As discussed in section 6.1.1, a futile cycle is reversal of product of a biochemical pathway by another opposite pathway. However, if only one pathway predominates at a time, the futile cycle can be avoided. This is more important when two competing pathways are operating operating within same compartment of a cell. One of the best mechanism to overcome such futile cycles is reciprocal regulation. The reciprocal regulation, is a mechanism of enzyme regulation by

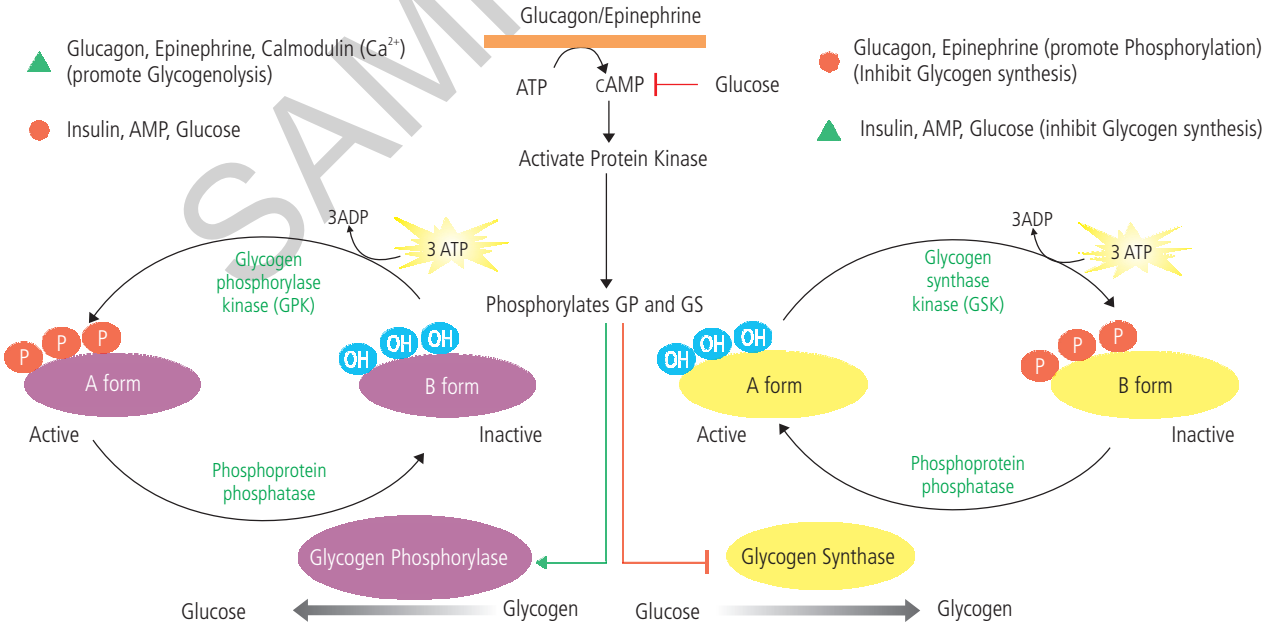


Fig 6.6 Reciprocal regulation of Glycogen metabolism by simultaneous phosphorylation of glycogen synthase and glycogen phosphorylase

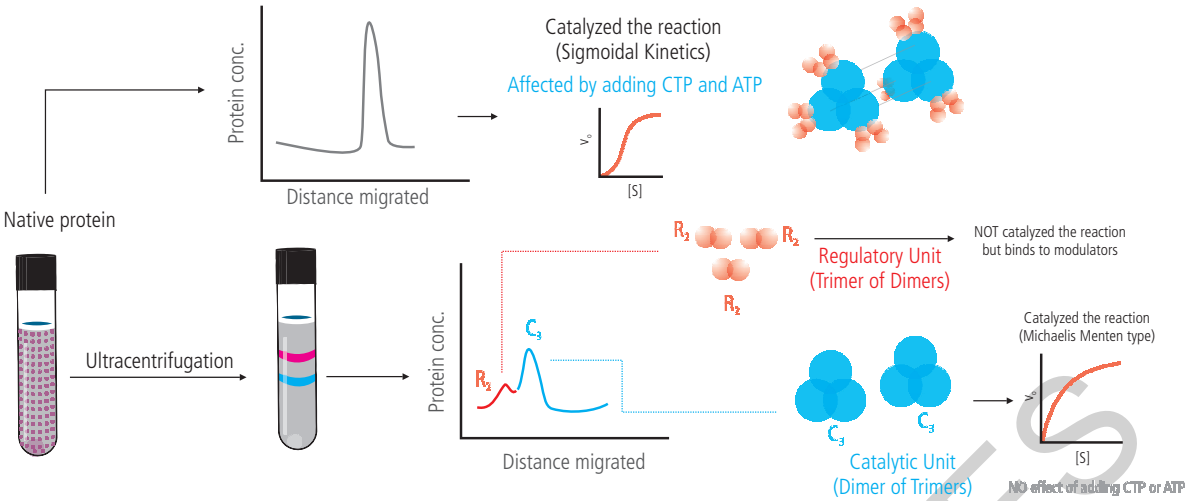
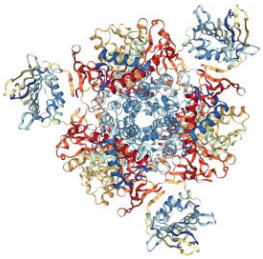


Fig. 6.7 Basic experiment to illustrate the salient features of allosteric enzymes using ATCase as model

composed of six catalytic chains, each with an active site, and six regulatory chains lacking catalytic activity. The catalytic subunits exist as a dimer of catalytic trimers, ($2xC_3$), while the regulatory subunits exist as a trimer of regulatory dimers, ($3xR_2$), therefore the complete holoenzyme can be represented as $(C_3)_2(R_2)_3$. The association of the catalytic subunits C_3 with the regulatory subunits R2 is responsible for the establishment of positive co-cooperativity between catalytic sites for the binding of aspartate and it dictates the pattern of allosteric response toward nucleotide effectors. Fig 6.7 illustrates basic strategy to demonstrate the allosteric nature of the enzyme.

3D Structure of ATCase
(PDB ID: 4KGV, NGL viewer)



6.3.2 Salient features of Allosteric Enzymes

Detailed analysis of such allosteric enzymes from different metabolic pathways has revealed the following common features among them.

- All allosteric enzymes are intracellular enzymes with no exception so far.
- Allosteric enzymes are usually present at the first committed steps of biochemical pathways.
- As they decide the overall rate of reaction of the whole sequence, they are also known as pacemaker enzymes.
- They are oligomeric and usually made up of 6-12 subunits (some exceptions exist – such as Glucokinase, which is monomeric.)
- The subunits are of two types i.e., catalytic and regulatory subunits. The catalytic subunits bind the substrates and catalyze the reaction whereas the regulatory subunits bind the modulators resulting in either increase or decrease of the rate of reaction.
- The catalytic and regulatory subunits are distinct proteins and are encoded by different genes.
- There are two types of binding sites on the enzyme, one substrate binding site (Isosteric site) and second modulator (Effector) binding site (Allosteric site)
- In absence of regulatory subunits, the enzyme behaves like a Michaelis-Menten enzyme.

6.3.3 Types of Allosteric Regulations

Based on the nature of modulator involved in regulation of the allosteric enzymes, the allosteric regulation may be classified as homotropic and heterotrophic regulation.

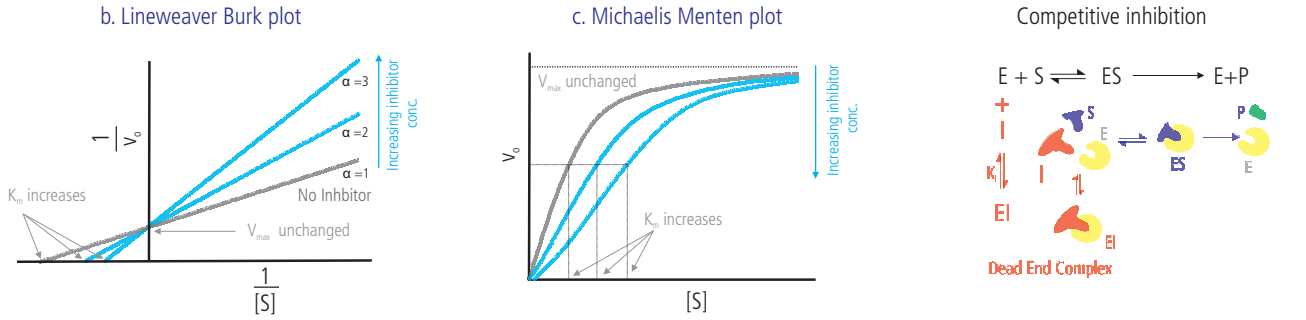


Fig. 6.11 Competitive Inhibition a. Mechanism of competitive inhibition b. Lineweaver Burk plot c. Michaelis Menten plot

Salient features of competitive inhibition are:

- Substrate competes with the inhibitor for the same site on the enzyme
- Both substrate and inhibitor bind to the same site
- EI complex does not lead to catalysis and known as dead end complexe.
- Apparent V_{max} = same as V_{max} (Not affected)
- Apparent $K_m = \alpha K_m$ (increased)
- Equation: $v_o = \frac{V_{max}[S]}{\alpha K_m + [S]}$ (alpha is defined in text above)
- Examples: Inhibition of succinate dehydrogenase by malonic acid (Malonate)

Note

In competitive inhibition, the Vmax does not change because increasing amounts of substrate can swamp the inhibitor (present in fixed concentration), allowing the enzyme to effectively not see the inhibitor at high substrate concentrations. On the other hand, the apparent K_m for competitive inhibition goes up because it takes more substrate to get the competitively inhibited reaction to $V_{max}/2$.

Practice Question

Question: If a competitive inhibitor called metrox was added to an enzyme pylinase, and the initial concentration of enzyme was 10 μM, concentration of inhibitor was 10 mM, concentration of substrate was 30 mM. The equilibrium constant for breakdown of EI complex was 10 mM s^{-1}. If the K_m of enzyme in absence of inhibitor was 0.5 mM, what will be the K_m (in mM) after addition of inhibitor?

Solution: In case of competitive inhibition apparent $K_m' = \alpha K_m$

And $\alpha = 1 + [I]/ K_i$

Hence, $\alpha = 1 + 10/10 = 2$

Hence new K_m (in presence of inhibitor) $= 2 \times 0.2 = 1$ mM

6.6.2 Uncompetitive Inhibition

Uncompetitive inhibition, on the other hand, does not involve the competition with substrate, but the inhibitor binds to enzyme substrate complex leading to an unproductive ESI complex. Infact, this occurs due to a conformational change in the enzyme after binding of substrate which created a new binding site for inhibitor. So in this type of inhibition one would not observe the EI complex in the reaction mixture.

The kinetics of ESI formation is based on the reaction between ES complex and Inhibitor. Hence, the amount of ESI complex formed in the reaction depends upon the concentration of inhibitor and reversibility of the reaction depends upon, dissociation constant for the breakdown of ESI complex (K_i'). The value of K_i' is calculated from the concentration of enzyme-substrate-inhibitor complex [ESI], inhibitor [I] and enzyme-substrate complex [ES] concentration respectively as follows

$$K_i' = \frac{[ES][I]}{[ESI]}$$

K_i and K_i'

Terms K_i and K_i' are used to differentiate the dissociation constants of EI and ESI complex respectively. K_i is used in case of competitive inhibition, while K_i' is used in case of uncompetitive inhibition. Some authors also use notations like K_i and K_{i-1} respectively.

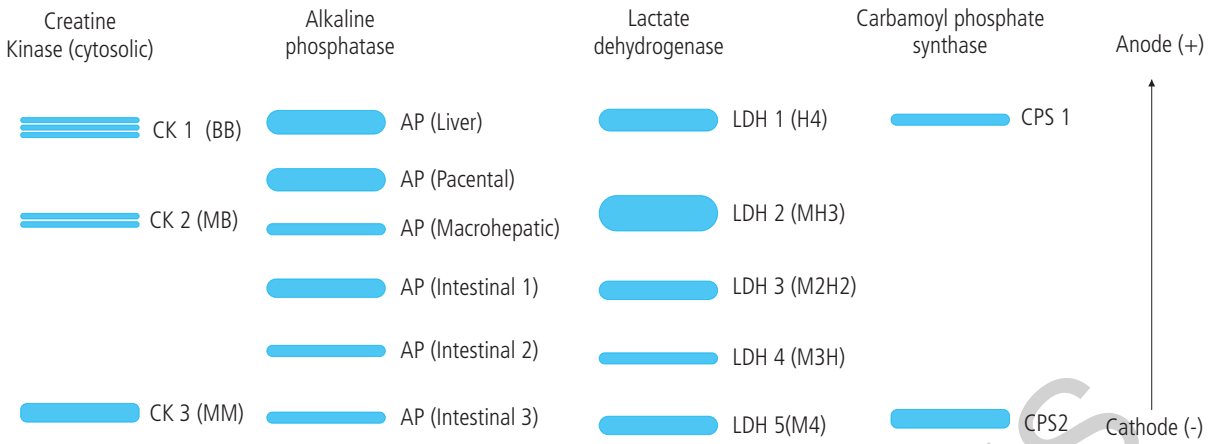


Fig 7.5 Electrophoretic mobility patterns of various isozymes. a. Creatine kinase isoforms, b. Alkaline phosphatase isoforms, c. Lactate dehydrogenase isoforms. Thicker bands represent relatively higher concentration in serum.

Practice Question

Question: Besides agarose gel electrophoresis that is commonly used at present, which other types of gel electrophoresis were used to separate isozyme in historical times?

Solution: Paper gel electrophoresis, cellulose acetate gel electrophoresis etc.

Note

Conventionally the electrophoretic pattern in gel electrophoresis is shown from wells on top to fastest lane at the bottom. However, as per the conventions of IUPAC-IUB, electrophoretic diagrams of isozymes are represented with anode on top (or right in case of horizontal figure). The most anodic isoform is assigned least number.

7.6.2 Other Techniques Used For Identification of Isozymes

Separation of most isoenzymes is accomplished by electrophoresis, column chromatography, or radioimmunoassay. Most clinical laboratories use electrophoresis on agarose gel or cellulose acetate combined with band quantification by fluorometric or spectrophotometric techniques. Although electrophoresis is possibly less sensitive than column chromatography or radioimmunoassay, there has been extensive experience and it is adequate for routine clinical use. The sensitive column chromatography method is more frequently used in research applications. Radioimmunoassay methods for isoenzymes can be accomplished rapidly and remains most sensitive method. Yet another assay used to identify the specific enzyme on the gel is known as zymography, which involves the mixing of substrate in gel specific for the enzyme in question under native or non-reducing, non-denaturing conditions. Enzymes on the gel shows an activity, which enables the user to detect the presence and relative abundance of the given enzyme.

7.7 SOME COMMON ISOZYMES

7.7.1 Lactate Dehydrogenase

Lactate dehydrogenase (LDH or LD) (EC 1.1.1.27) is an enzyme found in nearly all living cells (animals, plants, and prokaryotes). LDH catalyses the conversion of lactate to pyruvic acid and back, as it converts NAD^+ to NADH and back. A dehydrogenase is an enzyme that transfers a hydride from one molecule to another. Lactate dehydrogenase is composed of four subunits (tetramer). The two most common subunits are the LDH-M and LDH-H protein, encoded by the LDHA and LDHB genes, respectively (refer Fig 7.3 for actual structures of M and H peptides). These two subunits can form five possible tetramers (isoenzymes): 4H, 4M, and the three mixed tetramers (3H1M, 2H2M, 1H3M). These five isoforms are enzymatically similar but show differ-

CHAPTER 8

Non Canonical Enzymes

Learning Objectives

- RNA as enzymes and RNA world hypothesis
- DNA as enzymes and Antibodies as enzymes
- Synthetic enzymes including nanozymes

INTERESTING FACT

Cerium oxide nanoparticles, have proven to be highly efficient inorganic material capable of catalyzing the biological reactions similar to superoxide dismutase and catalase. Their catalytic activity is reportedly beneficial as a potential antioxidant in several models of oxidative stress. Due to their efficient UV protecting abilities, cerium oxide nanoparticles are also being foreseen as prospective ingredient of cosmetics. However, it is interesting to note that most of these catalytic activities diminish if the size of particles is increased and turned into a bulk material.

8.1 NON-CANONICAL ENZYMES

Although, the term non-canonical enzyme has not been used in enzymology, yet we used here to represent the enzyme like molecules, which have been associated with catalytic reactions in biological systems in some non-conventional manner. Until now we learnt that, enzymes are usually proteins, but new concepts emerged several years after the establishment of enzymology particularly with a landmark discovery of **ribozymes** by Cech and Altman. Beyond ribozymes, **DNazymes** have also been reported. Interestingly, some antibodies, specific to the transition state complex are also able to catalyse the reaction, in a much specific manner compared to enzymes and they are named **abzymes**. More recently, it has been possible to create chemical entities that catalyses reactions like enzymes, and they are named **chemzymes or synzymes**, which lead to the convergence of chemical catalysts and biological catalysts. Also, a sub-set of synzymes, particularly size restricted molecules, which are reduced to nanoscale and show catalysis similar to biological reactions are known as **nanozymes**. Most of the areas of research in this domain are still very active and new additions are continuously being made. In this chapter we will discuss, about all these non-canonical enzymes in detail.

8.2 RIBOZYME

Ribozymes are RNA molecules that are capable of catalyzing specific biochemical reactions, similar to the action of protein enzymes. Thomas R. Cech and Sidney Altman shared the Nobel Prize in chemistry for their discovery of catalytic properties of RNA in the year 1989. The term ribozyme, however, was first introduced by Kelly Kruger et al. in 1982. Cech discovered that introns in a ribosomal RNA gene in Tetrahymena thermophile (a ciliated protozoan and a model organism) are catalytic in splicing events and Sidney Altman, found that tRNA molecules are processed in the cell via an enzyme called RNase-P, which is responsible for conversion of a precursor tRNA into the active tRNA. Some common examples of ribozymes include the hammerhead ribozyme, VS ribozyme, leadzyme and the hairpin ribozyme. However, a classification scheme of ribozymes has also be been proposed on the basis of their secondary structure and functions. Table 8.1 summarizes common classes of ribozymes.

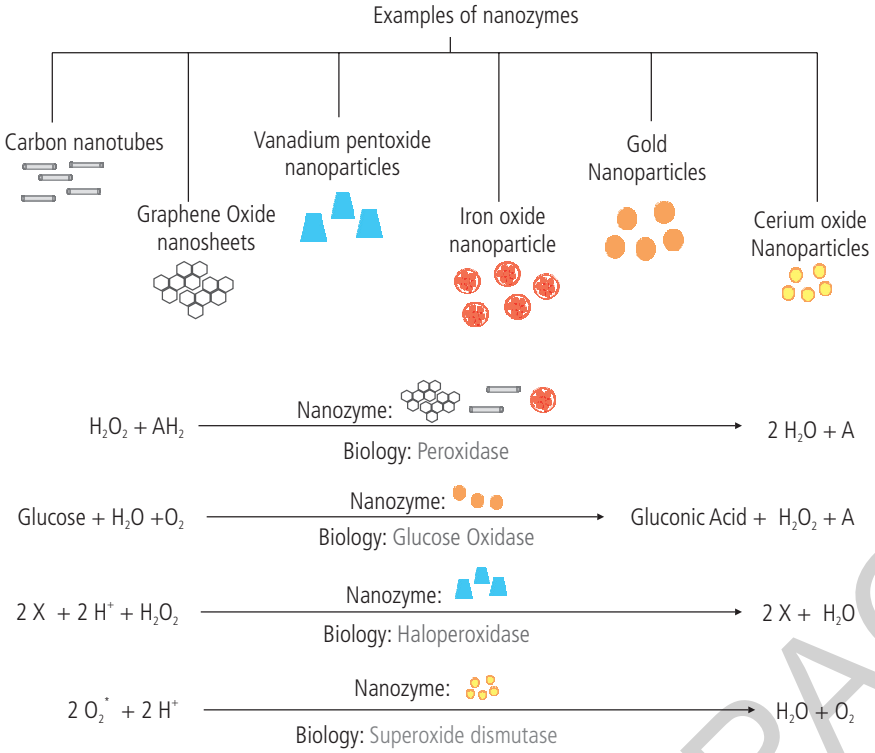


Fig 8.8 Different type of nanozyme and their potential mimicking actions in biological systems

Table 8.2 Comparision and contrast of some selective Nanozyme and corresponding enzymes

S.No.	Catalyst	V_{max}	K_m	K_{cat}
Oxidases and its mimetics				
1	MoO_3- TPP nanoparticles	1.13 μM min^{-1}	0.59 mM	2.78 s^{-1}
2	Polymer coated nanoceria	0.7 μM s^{-1}	3.8 mM	--
3	Native Human Su-Ox	--	0.017 mM	16 s^{-1}
Peroxidases and its mimetics				
1	Vanadium nanowires	0.28 M s^{-1}	0.4 mM	.065 s^{-1}
2	Hemin-Graphene	--	1.22 mM	246 min^{-1}
3.	Fe_3O_4 Nanoparticles	3.44 × 10^{-8} Ms^{-1}	0.098 mM	3.02 × 10^4 s^{-1}
4.	Glutathione peroxidase 1	--	10 mM	--

is generally agreed that the percentage of cerium atoms in the reduced state increases with decreasing particle size. It is observed that an increase in Ce^{3+} concentration from 17 to 44% as particle size decreased from 30 to 3 nm. With this reason scaling down the cerium oxide to nanosize proved beneficial in biological models. Another striking feature of the cerium oxide at nanoscale is that its lattice expands as the particles become smaller leading to decrease in oxygen release and re-absorption, therefore containing a larger fraction of reduced ceria atoms.

Well before the biological activity of cerium oxide was known, it begun to be used as an abrasive catalyst in chemical mechanical planarization/ polishing, as a catalyst in fuel cell power generation and catalytic converters, and in fuel borne additives. Studies demonstrating successful therapeutic or prophylactic effects of nanoceria have exponentially grown over the past decade. Aforesaid, switching between Ce^{3+} and Ce^{4+}oxidation states have been identified as

Benefits of Nanoceria During Hypoxia Stress

Based on emerging data and studies from around the globe, Arya et al (Author), hypothesized that nanoceria may have exemplary effects against hypobaric hypoxia induced oxidative stress. To this end, they synthesized spherical nanoceria particles (7-10 microns diameter) using microemulsion technology. These spherical nanoceria were then administered intra-peritoneally to male SD rats on a weekly basis for 5 weeks. Their lung tissue was harvested after an acute hypobaric hypoxia exposure at 25,000 ft (simulated). It was observed that nanoceria deposited in lung tissue and was effective at reducing ROS formation (possibly by quenching of radicals), glutathione oxidation, nitrosylation and carbonylation as well as inflammatory processes. In another study, the effects of nanoceria on mitochondrial membrane potential (MMP) was also studied and it was observed that cultured primary cortical cells from SD pups (1 day old) and challenged this cortical culture with high concentrations of hydrogen peroxide. Upon nanoceria application, it was observed, that nanoceria, lowered ROS and calcium flux in the culture. Moreover, nanoceria caused maintenance of MMP, restoration of NAD^+/NADH ratio and cellular ATP. All these together led to increased cell viability and reduced apoptosis. It hints at nanoceria as an effective agent in protecting brain against oxidative stress. But nanoceria have poor permeability through blood-brain barrier. To overcome this drawback, Arya et al, formulated a polyethyleneglycol (PEG) coating of nanoceria (PEG-CNP). The PEG-CNP efficiently localized in rodent brain. They observed, it not only abrogated oxidative stress but also prevented hypoxia induced loss of memory based cognitive function by augmenting neuronal survival. Another astonishing finding was the ability of PEG-CNPs to promote neurogenesis via a possible modulation in AMPK-PKC-CBP pathway. More recently, some of the promising activities of nanoceria such as augmentation of exercise induced mitochondrial biogenesis and UV protective abilities in skin keratinocytes have also been tested in lab by the authors.

primary events responsible for free radical scavenging in auto-catalytic manner, making nanoceria superlative to existing antioxidants. Oxygen defects in crystal lattice enables nanoceria to scavenge superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals and therefore depending upon surface oxidation states, nanoceria mimics the activities of cellular antioxidant enzymes, superoxide dismutase (SOD), catalase, oxidase, catalytic amplifier for alkaline phosphatase activities and peroxynitrite scavenging activities. Surface reactions underlying SOD and catalase mimetic activities have been illustrated in figure 8.9 below.

8.6.2 Potential Applications of Nanozyme

Development of biosensors: Nanozymes are being studied for the use in development of biosensors, such as H_2O_2 detection, which is of great interest owing to its important roles in biology, medicine, food industry and environmental protection. Besides, H_2O_2, biosensors for glucose, metal ions and DNA are also being developed.

Cosmaceutical applications: Unique UV absorbing properties of nanozyme such as cerium oxide, titanium oxide and zinc oxide have been widely studies and some of them also been commercialized. Nanozymes with UV protecting abilities and abilities to confer the scavenging of radicals are potentially important for the development of anti-ageing and sunscreen related cosmetic products.

improved significantly. Infact, artificial enzymes have also been synthesized successfully and represent the frontiers of enzyme technology.

In the following section we will discuss the various processes of enzyme technology and some commercial applications.

9.2 EXTRACTION AND PURIFICATION OF ENZYMES

Commercial production of enzymes is much different from the lab-scale synthesis, due to the fact that industrial production should be cost effective and handling of larger volumes and processes needs automation. The initial steps of the enzyme purification begin laboratories with the establishment of enzyme producing strain of a microbe. In earlier days extraction of organs/tissues and their homogenization was done, but now most of the enzyme can be genetically engineered and may be produced in various host systems. After the establishment of culture and characterization of stain, the scaling up of the process is done that requires a set up called bioreactor. The science of producing and designing biological products at commercial levels is known as **bioprocess technology** or **bioprocess engineering**, which requires use of principles of physics, mathematics, electronics and computer sciences besides biology of microbes. In most of the commercial processes a crude extract is first obtained by either breaking the cells (if the enzyme is intracellular) or collecting the culture media (if the enzyme is extracellular). The process of further processing and purification is also referred to as downstream processing in bioprocess technology. Fig 9.1 illustrates the basic steps of commercial enzyme production.

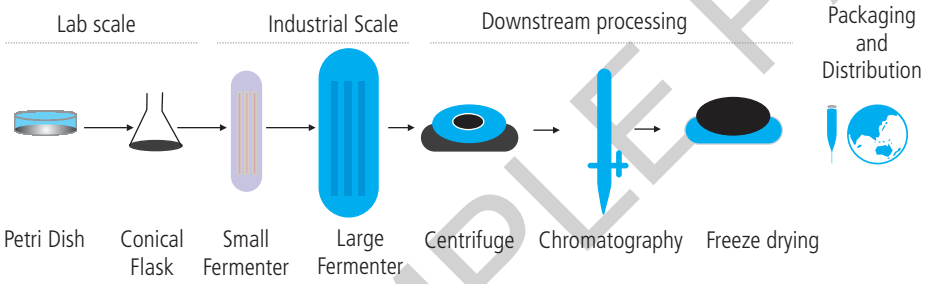


Fig 9.1 General outline of commercial production of enzymes, laboratory scale, industrial scale and downstream processing

9.2.1 Methods of Cell Disruption

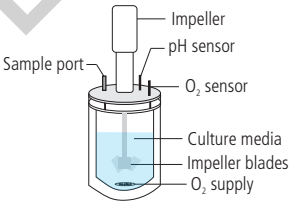
In case of intracellular localization of enzyme within the source, cell disruption is the first step of enzyme extraction. As enzymes are proteins, utmost care must be taken during the entire process to prevent the damage to original structure of protein to preserve the enzyme activity. Heat, shear, presence of proteases, foaming, oxidation and heavy metal toxicity are some of the common causes of premature damage to enzyme. The cell disruption methods can be grouped into two broad categories i.e. mechanical and non-mechanical methods. Mechanical methods are cost effective for the large scale applications hence they are often used in industries. While, at the laboratory scale the cell disruption can be performed using variety of non-mechanical methods that includes, physical methods such as osmotic shock or thermolysis, chemical methods such as detergent based lysis or enzymatic methods (Fig 9.2).

Some of the industrial methods of cell disruption are discussed below in more detail:

Bead mills: Bead mill disrupts the cell suspensions by agitating them in a grinding cylinder. The grinding cylinder is filled with the beads made up of glass, alumina, titanium carbide, zirconium oxide or zirconium silicate (usually 0.2 -.1.0 mm diameter). Cells are broken by the high liquid shear gradients and collision with the beads. Bead mills can handle a volume from

Taka-diastase

The first enzyme produced at the industrial scale was **Taka – diastase,** a fungal amylase, in the year 1896 to cure digestive disorders. Jokichi Takamine was awarded a patent for commercial production of this enzyme. Later in 1913, in France, Boidin and Effront produced an extremely heat-stable alpha-amylase using Bacillus subtilis, when grown in still cultures on a liquid medium prepared by extraction of malt or grain.



Novozyme

In 1925 the brothers Harald and Thorvald Pedersen founded Novo Terapeutisk Laboratorium and Nordisk Insulinlaboratorium with the aim to produce insulin. In 1941 the company's predecessor launched its first enzyme, trypsin, extracted from the pancreas of animals and used to soften leather, and was the first to produce enzymes by fermentation using bacteria in 1950s. In the late 1980s Novozymes presented the world's first fat-splitting enzyme for detergents manufactured with genetically engineered microorganisms, called Lipolase.

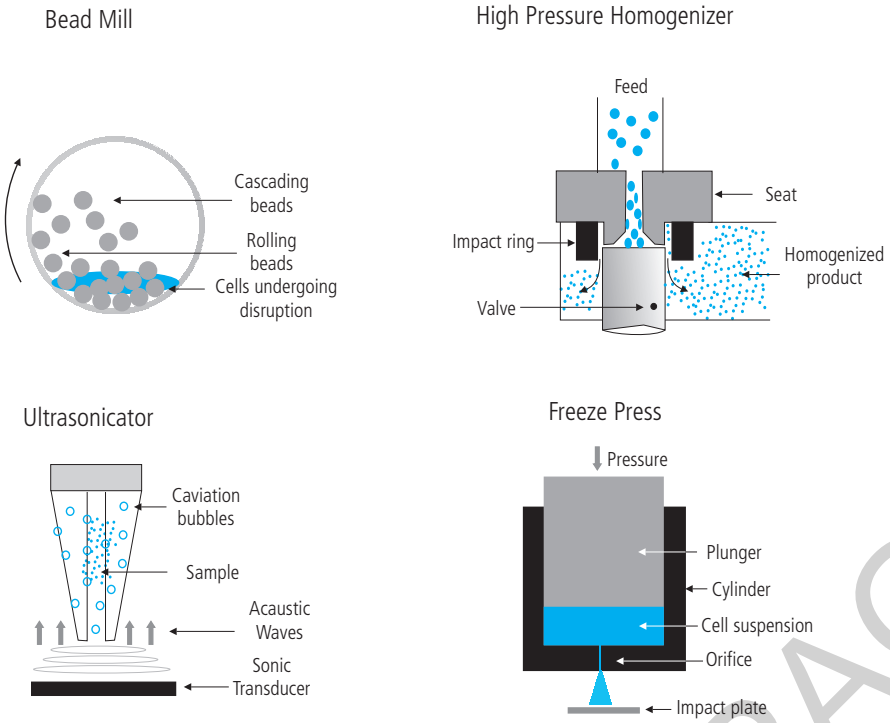


Fig 9.3 Common mechanical methods of cell lysis, a. Bead mill, b. High pressure homogenizer, c. Ultrosonicator, d. Freeze press

Here, C_{max} is the concentration of product that can be released from a given amount of cell suspension, C is the concentration of product released at a given time "t" and k is the first order rate constant. This relationship holds true only for batch mode of operation. The value of k depends on type of impeller, bead size and loading, speed of agitation and temperature. , In case of high pressure homogenization, t is replaced by N, where N is the number of passes through the valve.

Question: How to calculate the stress developed on cell while using high pressure homogenization?

Solution: Cell passing through a small valve experience stress developed within the fluid and as result it get disrupted. The stress developed is expressed as dynamic pressure Ps and it is expressed as $P_s = \frac{1}{2}\rho V^2$

P_s is dynamic pressure, v is jet velocity and p is the density of fluid. Cell disruption in high pressure homogenizer and release of a produce is a first order kinetics and it may be given by first order equation

9.2.2 Methods of Enzyme Purification

After the cells have been disrupted or culture media has been collected (in case of secreted enzyme) the lysate may contain a number of components other than the desired enzymes. The process of enzyme extraction from this mixture and final purification is a tedious job. The lysate that is obtained from the source is known as **crude extract**. The volume is significantly reduced during the process due to the loss of impurities in subsequent steps with a concurrent increase in the specific activity of enzyme (described later in this chapter). There are several methods of enzyme purification from the crude extract. Fig 9.4 represents the outline of various strategies used for the extraction of enzymes, based on their different features.

purification methods is also very critical. If one begins with affinity chromatography in crude extract the columns may be clogged and lead to poor yield. In general, the sequence of events of purification is as follows (Fig 9.5):

- Mincing or chopping of tissue or homogenization of cells
- Lysis of cells using lysis buffer
- Precipitation of proteins using ammonium precipitate (an ultracentrifugation separation can also be performed).
- Ion exchange chromatography (cation exchange or anion exchange or both types may be used sequentially)
- Size exclusion chromatography (size based separation)
- Affinity purification (separation on preparative polyacrylamide gel can also be performed)

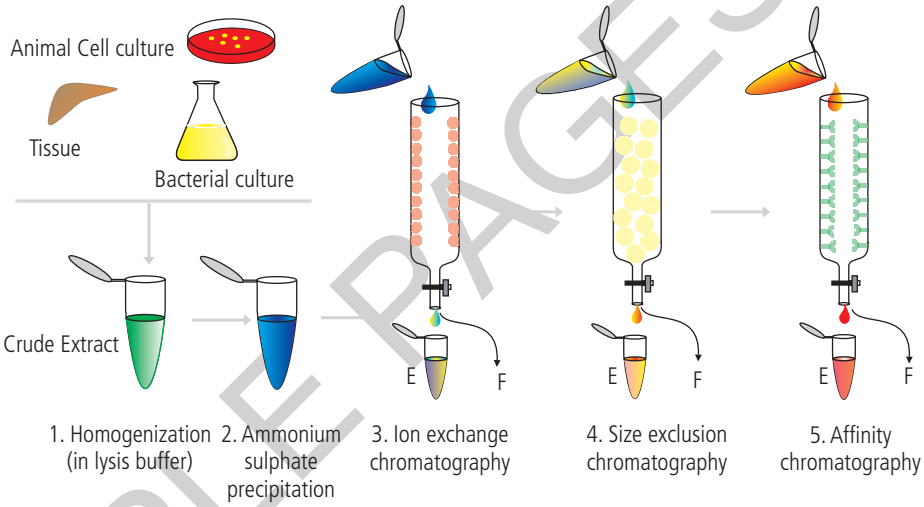


Fig 9.5 Sequence of enzyme purification steps

9.3 ASSESSING THE QUALITY OF ENZYME PURIFICATION

During the various steps of enzyme purification, it is essential to evaluate the degree of purification, various parameters that are used to evaluate the quality of each purification steps are described in following subsections. Also, there are three basic terms that are used to define the **purification, yield, enzyme activity and purity**. They have been described in brief below. In order to assess the quality of purification, it is also important to know that the initial fraction of enzyme, which is directly isolated after the cell lysis, homogenization of tissue or media from a bioreactor is known as **crude extract,** while the collected extract after subsequent purification steps is known as **purified fraction.**

Activity vs Conc.

In a solution of pure enzyme, the concentration of protein is equivalent to concentration of enzyme. However, if the solution is impure like crude extract, the protein concentration may not represent the enzymes. Hence, in such conditions, the term enzyme activity is prefered.

9.3.1 Total Enzyme Activity

While we are assessing the purity or catalytic efficiency of enzyme in solutions, it also contains several components other than enzyme, it is not possible to predict the exact number of moles of enzyme in a sample. Hence, as an alternative means, term activity was introduced. Activity is the indirect representation of enzyme concentration in a reaction mixture. For the quantitative determination of enzyme activity, initial rates are measured at different enzyme concentrations and near substrate saturation, in a suitable temperature range (25-37^{o}C) and at optimal pH. In a certain range the enzyme activity is proportional to the enzyme concentration. The enzyme

Various techniques such as error-prone PCR, cassettes mutagenesis, DNA shuffling, mutator strains and recombination were used to create genetic diversity. The process of screening of mutants involved several routine analytical methods such as mass spectrophotometry, high-performance liquid chromatography followed by either medium throughput strategies such as Liquid cell culture in microtitre plates, colonies on solid medium or high throughput methods such as phage display, cell surface display followed by fluorescence-activated cell sorting, , in vitro compartmentalization assisted with microfluidic-based screening and micro capillary single-cell analysis and laser extraction methods. In this manner, the evolved gene product is evaluated and either selected for use or allowed to undergo repeated cycles of evolution until desired features are obtained.

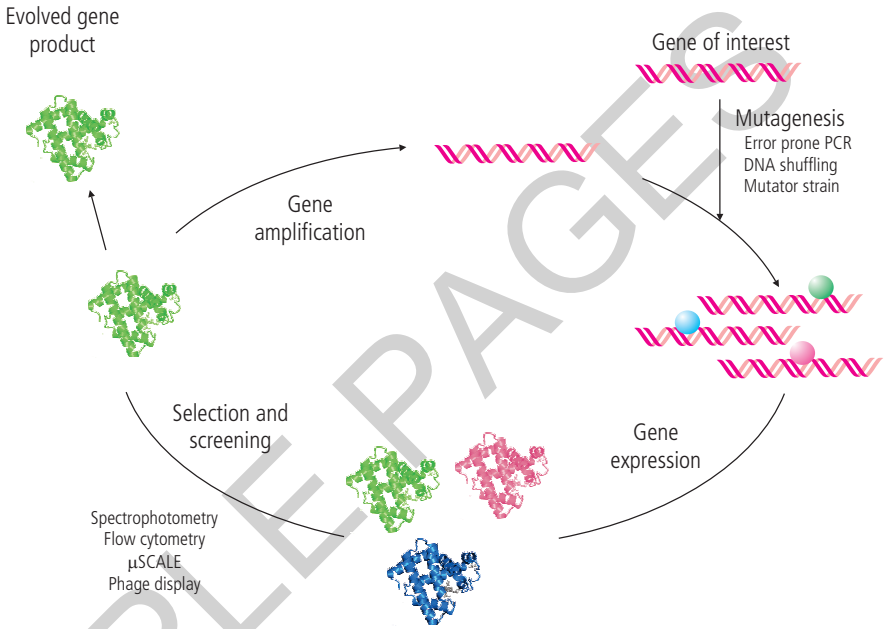


Fig 9.8 Outline of sequence of events in directed evolution of enzymes (Redrawn after Hivert et. al., 2018)

It is not possible to randomise every position in an enzyme, usually peptides not larger than 200-300 amino acid residues are preferred. Arnold and co-workers have shown by many examples that library design must be based on molecular insight and knowledge-based choices of which amino-acid positions to vary, combined with some element of added randomness, e.g. through error-prone PCR. A prominent early contributor to the development and implementation of methodology for directed evolution was the late William (Pim) Stemmer. Stemmer introduced a DNA recombination strategy termed "DNA shuffling" to the evolution of enzymes. This was an efficient way to propagate beneficial mutations while increasing the size of a DNA library.

Directed evolution of enzymes is highly useful process in enzyme engineering with a broad spectrum of commercial and research based applications in various sectors such as developing new chemical reactions, biofuel production, metabolic engineering, enantio-selectivity and organic synthesis.

9.6 COMPUTATIONAL ENZYME DESIGN

Computational enzyme design is conceptually similar to catalytic antibody technology (Abzyme synthesis, as discussed in chapter 8) but has much higher potential. Unlike abzyme generation where we utilize an imperfect transition-state analog to provide chemical instruction, computational design begins with the quantum mechanically calculated structure of the rate-limiting

transition state(s) of the target reaction. Amino acid side-chain surrogates are explicitly included in the calculations as functional groups to stabilize this high-energy species. The resulting ensemble represents an idealized three-dimensional model of a minimal active site, also called a theozyme (Chapter 8, Section 8.10), which is docked in silico into structurally characterized proteins from the Protein Data Bank using programs such as RosettaMatch, ORBIT or Scaffold Select. In addition to identifying a sterically complementary fit, the ends of the catalytic groups have to be connected to the protein backbone. Residues in and around this pocket are then redesigned for optimal packing of the transition state and catalytic groups. The design algorithms iteratively search the conformational space of the ligand and the side chains to minimize the energy of each possible sequence. This process mimics antibody affinity maturation in the immune system, with the calculated stability of the fold serving as a selection criterion for passing a design on to the next round of optimization. Promising designs, ranked according to their calculated energies, are then tested experimentally. In more specific terms, there are three general steps in computational enzyme design, first a theozyme model is constructed based on computer algorithms and existing knowledge of catalysis, second, a set of proteins of known structure collected. Second, these protein scaffolds are searched for sites where amino acids can be built off the native backbone and interact with the transition state as defined in the theozyme. Finally, the non-catalytic residues in the pocket are redesigned to further refine substrate, and transition state binding ability. Fig 9.9 illustrated a brief outline of the process.

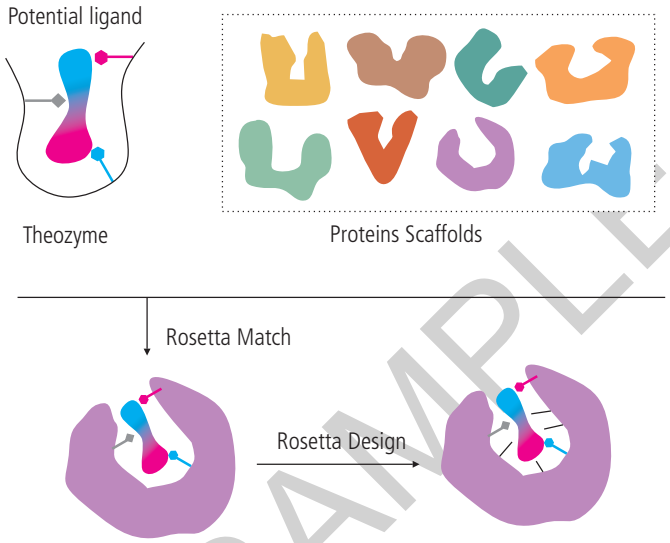


Fig 9.9. Outline of computational enzyme design

Table 9.6 Comparison between kinetic parameters of natural, designed and optimized enzymes

	Natural Enzymes	Computational enzymes (un-optimised)	Computational enzymes (optimised)
k_{cat} (s^{-1})	1.1×10^{2}	1.5×10^{-4}	1.5×10^{-2}
K_m (M)	9.7×10^{-7}	5.7×10^{-4}	2.0×10^{-4}
K_{tx}^{-1}	5.2×10^{18}	8.3×10^{6}	4.7×10^{9}
K_{cat}/k_{uncat}	1.0×10^{16}	4.2×10^{3}	5.9×10^{5}
All the values are representing the median observed under standard conditions, based on data from Siegel et al, Current Opinion in Structural Biology 2014, 27:87–94. K_{tx}^{-1} represents transition state constant.			

Computational tools for Enzyme Design

S. No.	Tool/Db	Application
1	PDB	Structure comparison
2	FATCAT	
3	MATRAS	
4	PDBeFold	
5	CASTp	Catalytic site
6	SABER	
7	AutoDock	Docking
8	Gold	
9	ENCoM	Ensemble
10.	ROSETTA	De novo design
	Orbit	

9.6.1 Computational tools used in enzyme design

A large number of computational tools including software packages and databases are required for computational enzyme design. Some of the most popular primary databases used to obtain preliminary information are Uniprot and RCSB (Research collaborator for structural biology), which contain information about the protein sequence and structures of proteins, including enzymes. Many different web services are available for structural comparison of a structure of interest against the PDB database, including Dali, FATCAT, MATRAS and PDBeFold. After structure comparison, the catalytic site prediction can be performed using an excellent web-tool called CASTp, which helps in interpreting the function of active-site residues and shed light on the catalytic mechanism. Software suites ROSETTA and ORBIT are the most widely used. De-signed enzymes can catalyze non-biological reactions. Besides this, several new tools are rapidly emerging up and the concept of deep learning and artificial intelligence has also begun to be used in enzyme design, which is beyond the scope of this text and can be explored in standard monographs.

QUICK REVISION

- Enzymes have a very large and profound global market and therefore commercial production is important.
- The process of commercial production is different from lab extraction methods.
- Enzyme purification from crude extract require a multi-step separation, in which the order or sequence of procedures is also critical.
- Enzyme immobilization is the common technique for effective packaging, transport and industrial utilization of enzymes.
- Enzymes can be modified at gene level by performing site directed mutagenesis.

QUESTIONS

1. One gram of fresh tissues from a liver of rat contained 40 units of purified enzymes lactate dehydrogenase, with turnover number of 6×10^4 min^{-1}. Calculate intracellular concentration of enzyme? (Provided that one gram of liver tissues contains about 0.8 ml of intracellular water).
2. Champakali, a biochemist was assigned an enzyme purification problem in her interview for a bioprocess company. She was told that a crude cell-free extract of heart tissue contained 32 mg/ml protein. Ten microliter of the extract catalysed a reaction at the rate of 0.14 µmol/min under optimal conditions. Fifty microliter of the extract was fractionated using ammonium sulphate precipitation. The fraction precipitating between 20% and 40% saturation was re-suspended in 10 ml. The fraction was found to contain 50 mg/ml protein. Ten microliters of this purified fraction catalysed the reaction at the rate of 0.65 µmol/min. she was then asked to calculate the percentage recovery of the enzyme in purified fraction and degree of purification obtained. Can you help her to solve this problem?
3. What is meant by unipontal and multipontal covalent linkage used for enzyme immobilization?
4. In order to carry out site-directed mutagenisis, a DNA primer was required to be designed. Consider that a protein named Jinni need to be made much faster by replacing histidine to leucine. Wild type sequence of protein catalytic site is Tyr-Leu-His-Val, corresponding to a genetic sequence of UACCUGCACGUC. Can you determine the type of primer that may be used to create the mutation?
5. How site directed mutagenesis can be used to evaluate the catalytic triad present in enzymes?

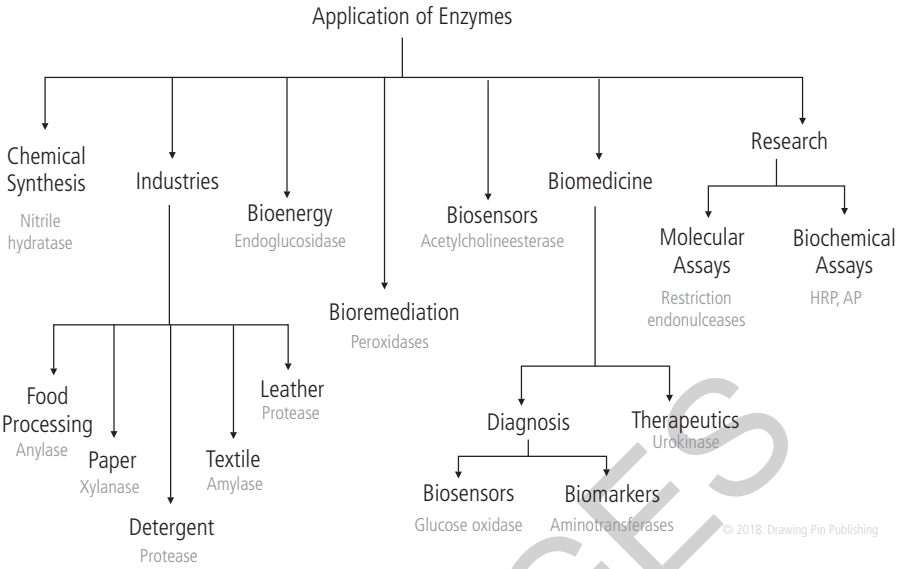


Fig10.1 Summary of applications of enzymes: various domains of enzyme application with examples.

cephalosporanic acid (7-ADCA). To further augment the potency of penicillins and broaden their antimicrobial range, the first generation penicillins are also used as precursors for semisynthetic penicillins. The enzyme penicillin G acylase is most widely used enzyme for the biotransformation of penicillin G to semisynthetic penicillin or cephalosporin (other important antibiotics).

10.2.2 Acrylamide Synthesis

Biotransformation

Biotransformation is the chemical modification (or modifications) of various molecules including nutrients, amino acids, toxins, and drugs etc. Biotransformation cab be carried out by an organism on artificially, using a synthetic process.

Bulk chemical synthesis is the large scale production of commercially important chemicals. Until recently enzymes were not much used in this segment of industry but the emergence of protein engineering and novel selection tools based on metagenomic library and in silico genome databases made usages of enzymes advantageous. One of the most successful stories in enzyme technology came from hydration reaction of nitrile group leading to the formation of acrylamide. Hideaki Yamada and his research team at the Kyoto University of Japan screened and developed a process based on nitrile hydratase of *Rhodococcus rhodochrous*. Similar process to convert 3-cyanopyridine (nicotine nitrile) for the production of nicotinamide was developed based on same nitrile hydratase.

10.2.3 Polymer Synthesis

Enzyme-catalyzed polymerization or enzymatic polymerization refers to in vitro polymerization by non-biosynthetic pathway using an enzyme as a catalyst. The environmental toxicity caused by enzymatic polymerization is much less compared to the use of the conventional toxic metal catalysts. In many cases, the starting materials are natural resources such as biodegradable as well as environment-friendly polymer materials which can be utilized to produce polymer material through enzymatic catalysis. That is why enzyme is also considered as a green chemical catalyst in the polymerization, resulting in the acceleration of the recirculation of material obtained from the natural world. The formation of a polymer by the oxidation of phenols occurs in the natural polymer such as lignin and melanin through the catalysis of enzyme. Since the mid-1980s, the horseradish peroxidase (HRP) has been used to polymerize the variety of phenols.

10.8.2 Use of Enzymes in Biochemical Assays

A plethora of biochemical assays, including immunological, histochemical assays are routine procedures in basic biology research which extensively involve enzymes. Most enzymes that are used in these assays have a primary function of catalysing a detectable reaction, i.e generation of a colour or light that can be measured using spectrometric or photometric devices. In some cases, when the enzymes are not directly associated with the chemical reaction being detected, the reactants or metabolites of the reaction are utilized to couple them to another reaction which can be linked to specific enzyme that gives detectable signal, this process is known as enzyme coupling. Some commonly used methods and respective use of enzymes have been described below:

Enzyme Linked Immunosorbent assay (ELISA): Enzyme linked Immunosorbent Assay, as the name suggests involve linking of enzymes with immunological molecules such as antibodies. ELISA is based on two fundamental principles, one ability of antibodies or antigens to get immobilized on a polystyrene or similar surface, and their subsequent detection with the help of a chromogenic reaction catalyzed by antigen. High specificity due to use of antibodies and good sensitivity due to chromogenic reactions make ELISA, widely used technique both in laboratories and clinics. Most commonly used enzymes in ELISA are horshradishperoxidase (HRP), alkaline phosphatase (AP) and beta galactosidase. Enzyme beta-lactamase, though introduced as a label in late seventies has not yet become very popular inspite of having the necessary features of an enzyme to be used in ELISAs.

Stop Solution

The colour is produced by HRP (and enzyme) and TMB (usual substrate) continue to proceed until all substrate is consumed which is added in great excess, therefore in order to note the visual differences it is important to stop the reaction by adding stop solution, so that signal from all the wells does not become same. A stop solution usually contains acid.

Immunoblotting: The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Immunoblotting is antibody based detection of protein transferred onto the membrane. Immunoblotting can produce qualitative and semi-quantitative data about the protein of interest. Most commonly used enzymes which are conjugated to the detection antibody are horseradish peroxidase (HRP) or alkaline phosphatase (AP).

Immunohistochemistry: Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The antibody-antigen binding can be visualized in different manners. Enzymes, such as horseradish Peroxidase or alkaline Phosphatase are commonly used to catalyze a color-producing reaction.

Practice Question

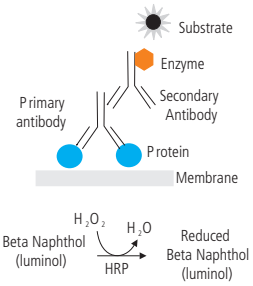
Question: Name the source of enzyme horseradish peroxidase (HRP)?

Solution: The enzyme HRP is isolated from the roots of horseradish plant (*Armoracia rusticana*)

10.8.3 Chromogenic Reaction by Enzymes in Various Assays

Colour development in ELISA is a result of reaction catalyzed by enzyme conjugated to secondary antibody. As the number of antibodies bound will be proportional to the amount of antigen, the intensity of this colour is a direct measure of antigen concentration (inverse in case of competitive ELISA). Two most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase. Less commonly, beta-galactosidase and urease can also be used. These enzymes can catalyze a reaction that converts a substrate to coloured compound (chromogenic). The intensity of colour is then measured by spectrophotometry to estimate the quantity of antigen e.g alkaline phosphatase uses paranitrophenolphosphate (pnpp) and turns into a yellow green compound and beta galactosidaase converts o-nitrophenol beta galactoside (ONPG) into

Luminogenic Reaction



APPENDIX 3

Regulation of Enzymes

(Key Regulators of Important Biochemical Pathways)

S.No	Name of enzyme	Positive regulator	Negative regulator	Comment
Glycolysis				
1.	Hexokinase	AMP / ADP	Glucose-6-phosphate	Feedback inhibition
2.	Phosphofructokinase I	Fructose 2,6-bisphosphate , AMP	Citrate, ATP , PEP	PFK II forms F2,6BP while FBase degrades
3.	Pyruvate Kinase	Fructose 1,6 bisphosphate	ATP, Alanine, Acetyl CoA	Phosphorylation of PK causes its inhibition
TCA cycle				
4.	Pyruvate dehydrogenase complex	Fructose 1,6 bisphosphate, AMP, CoA, NAD^+, Ca^{2+}	NADH, Acetyl CoA, ATP	Phosphorylation also inhibits PDH (calcium activates phosphatases)
5.	Citrate synthase	ADP	NADH, Succinyl CoA, Citrate, ATP	
6.	Isocitrate dehydrogenase	Ca^{2+}, ADP	ATP, NADH	
7.	α-ketoglutarate dehydrogenase	Ca^{2+}	Succinyl CoA, NADH	
Gluconeogenesis				
8.	Pyruvate Carboxylase	Acetyl CoA	ADP	Stimulated during fasting and diabetes
9.	Fructose 1,6 bisphosphatase	Citrate	AMP, Fructose 2,6-bisphosphate	Stimulated during fasting and diabetes
Glycogenesis				
10.	Glycogen Synthase	Glucose, ATP, Glucose-6-phosphate	Ca^{2+}	Dephosphorylated form is active form
Glycogenolysis				
11.	Glycogen Phosphorylase	Ca^{2+}	Glucose, ATP, Glucose-6-phosphate	Phosphorylated form is the active form
Pentose Phosphate Pathway				
12.	Glucose-6-phosphate dehydrogenase (G6PD)	$NADP^+$	NADPH Acetyl CoA	SIRT2-mediated deacetylation and activation of G6PD
Fatty Acid Biosynthesis				
13.	Acetyl CoA Carboxylase	Citrate	Palmitoyl CoA	Dephosphorylated from is active
Beta Oxidation				
14.	Carnitine Palmitoyl Acyltransferase I		Malonyl CoA, ATP, NADH	AMPK inhibits at trancriptional level
Cholesterol Biosynthesis				
15.	HMG CoA Reductase		Mevolanate	Compactin, lovastatin are artificial drugs (competitive inhibitors), dephosphorylated from is active.

S.No	Name of enzyme	Positive regulator	Negative regulator	Comment
Ketone Bodies Biosynthesis				
16.	HMG CoA Synthase *	Acetyl CoA	Succinyl CoA	Covalent modification by succinylation inhibits and desuccinylation activates enzyme.
Purine Biosynthesis				
17.	PRPP Synthetase	PRPP	ATP, AMP, GTP, GMP	Two different allosteric sites for AMP and GMP
Pyrimidine Biosynthesis				
18.	Aspartate transcarbamylase (ATCase)	ATP	CTP	
19.	Cabamoyl Phosphate Synthase II	ATP	UTP, UDP, dUTP, CTP	CPS I is involved in urea cycle
20.	OMP Synthase		UMP, CMP	
21.	CMP Synthase	GTP	CMP	
Urea cycle				
22.	Carbamoyl phosphate synthase I	N-acetylglutamate		Acetyl CoA and glutamate are essential for synthesis of NAG, by enzyme NAG synthase

* HMG CoA synthase has two isoforms, cytosolic form involved in cholesterol biosynthesis and mitochondrial isoform involved in ketone bodies biosynthesis.

Most of the regulations enlisted in the table are based on regulation by metabolites. However, hormonal control via glucoagon, insulin, epinephrine, cGMP etc. also exits that involves colvalent modification and activation/deactivation of most of the enzymes.

Solutions of Selected Questions

Chapter 1

Q1. We can use following equation to solve the question,

$$\ln\frac{k_1}{k_2} = -\frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

$$\ln\frac{3.4}{31} = -\frac{E_a}{8.314}\left(\frac{1}{600} - \frac{1}{750}\right)$$

$$(1.223 - 3.40) = -\frac{E_a}{8.314}\left(\frac{50}{600\times750}\right)$$

$$E_a = \frac{2.17\times8.314\times750\times600}{50}$$

$= 162372.42$ Joules mol^{-1} or 162.37 kJ mol^{-1}

Q2. Substitute the numbers into the equation,

$$\ln k = -\frac{Ea}{RT} + \ln A$$

$$\ln k = -\frac{200\times1000}{8.314\times289} + \ln 9$$

$k = 6.37\times10^{-36}\ M^{-1}s^{-1}$

Q3. Use the relationship

$$\ln\frac{k_1}{k_2} = -\frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

$$\ln\frac{7}{k_2} = -\frac{900\times1000}{8.314}\left(\frac{1}{370} - \frac{1}{310}\right)$$

$k_2 = 1.788\times10^{-24}\ M^{-1}s^{-1}$

Q4. Use the equation, $k = Ae^{\frac{-E_a}{RT}}$

$$12 = 15\times e^{\frac{-E_a}{8.314\times22}}$$

$Ea = 40.82$ J/mol

Q5. Use the equation $\ln(k_1/k_2) = -Ea/R(1/T_1 - 1/T_2)$

$\ln(15/7) = -[(600\times1000)/8.314](1/T_1 - 1/389)$

$T_1 = 390.6K$

Chapter 2

Q1. There are exceptions to this general rule. Some are justified because the mechanism of the reaction or the substrate specificity is so different as to warrant different entries in the enzyme list. This applies, for example, to the two cholinesterases, EC 3.1.1.7 and 3.1.1.8, the two citrate hydro-lyases, EC 4.2.1.3 and 4.2.1.4, and the two amine oxidases, EC 1.4.3.4 and 1.4.3.6. Others are mainly historical, e.g. acid and alkaline phosphatases (EC 3.1.3.1 and EC 3.1.3.2).

Q2. The naming of enzyme was done based on the experimental evidences in some cases, however, sometimes those enzymes were catalysing reverse reaction in physiological systems. As an extension of third principle of IUCMB, the direction chosen should be the same for all enzymes in a given class, even if this direction has not been demonstrated for all. Thus the systematic names, on which the classification and code numbers are based, may be derived from a written reaction, even though only the reverse of this has been actually demonstrated experimentally. In the list in this volume, the reaction is written to illustrate the classification, i.e. in the direction described by the systematic name. However, the common name may be based on either direction of reaction, and is often based on the presumed physiological direction.

Chapter 3

Q1. Mn^{2+} are paramagnetic while Mg^{2+} ions are diamagnetic and various techniques involving the study of enzyme metal complex, involve the electron spin, hence Mn^{2+} gives a better signal due to paramagnetic nature. Therefore, it is easy to detect Mn^{2+}, compared to Mg^{2+} in enzymes.

Q2. Mg^{2+} is accumulated inside the cells in exchange of Ca^{2+} ions in opposite direction. Therefore, it is expected that enzymes requiring Ca^{2+} are mainly extracellular.

Bibliography:
Cited text and further readings

Acquaah G, 2009. Principles of plant genetics and breeding, John Wiley & Sons.

Athel C-B. Understanding allosteric and cooperative interactions in enzymes. The FEBS Journal 2014;281(2):621–632.

Berg JM, Tymoczko JL, Stryer L, 2008. Biochemistry (Loose-Leaf), Macmillan.

Boyce STipton KF. Enzyme classification and nomenclature. eLS 2001.

Bugg TD, 2012. Introduction to enzyme and coenzyme chemistry, John Wiley & Sons.

Cech TR. RNA as an enzyme. Scientific American 1986;255(5): 64–75.

Charnock SJMcCleary B. Enzymes: industrial and analytical applications. Megazyme Rep 2006:1–5.

Cook PFCleland WW, 2007. Enzyme kinetics and mechanism, Garland Science.

Cornish-Bowden A, 2012. Fundamentals of enzyme kinetics, Wiley-Blackwell Weinheim, Germany.

Dahlquist F. [13] The meaning of scatchard and hill plots. Methods in enzymology: Elsevier; 1978. vol. 48, p. 270–299.

Damián-Almazo JYSaab-Rincón G. Site-directed mutagenesis as applied to biocatalysts. Genetic Manipulation of DNA and Protein-Examples from Current Research: InTech; 2013.

Eyers PAMurphy JM. The evolving world of pseudoenzymes: proteins, prejudice and zombies. BMC biology 2016;14(1):98.

Fersht A, 1999. Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding, Macmillan.

Greiner R, Silva LGd, Couri S. Purification and characterisation of an extracellular phytase from Aspergillus niger 11T53A9. Brazilian Journal of Microbiology 2009;40:795–807.

Gutfreund H. Rapid-flow techniques and their contributions to enzymology. Trends in biochemical sciences 1999;24(11):457–460.

Hammann C, Luptak A, Perreault J, de la Peña M. The ubiquitous hammerhead ribozyme. RNA 2012;18(5):871–885.

Hammes GGWu C-W. Kinetics of allosteric enzymes. Annual review of biophysics and bioengineering 1974;3(1):1–33.

Hill R, 1970. The chemistry of life: Eight lectures on the history of biochemistry, CUP Archive.

Hilvert D. Critical analysis of antibody catalysis. Annual review of biochemistry 2000;69(1):751–793.

Hollenstein M. DNA Catalysis: The Chemical Repertoire of DNAzymes. Molecules 2015;20(11):19730.

Horton HR, Moran LA, Ochs RS, Rawn JD, Scrimgeour KG, 1996. Principles of biochemistry, Prentice Hall Upper Saddle River, NJ.

IUPAC-IUB Commission on Biochemical Nomenclature (CBN). European Journal of Biochemistry 1971;24(1):1–3.

Jaffe EK. Morpheeins–a new structural paradigm for allosteric regulation. Trends in biochemical sciences 2005;30(9):490–497.

Khatkhatay MIDesai M. A comparison of performances of four enzymes used in ELISA with special reference to β-lactamase. Journal of immunoassay 1999;20(3):151–183.

Kitto GB, Wassarman PM, Kaplan NO. Enzymatically active conformers of mitochondrial malate dehydrogenase. Proceedings of the National Academy of Sciences 1966;56(2):578–585.

Klinman JPBonnot F. Intrigues and intricacies of the biosynthetic pathways for the enzymatic quinocofactors: PQQ, TTQ, CTQ, TPQ, and LTQ. Chemical reviews 2013;114(8): 4343–4365.

Koshland Jr D, Némethy G, Filmer D. Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 1966;5(1):365–385.

Laidler KJ. Symbolism and terminology in chemical kinetics. Pure and Applied Chemistry 1981;53(3):753–771.

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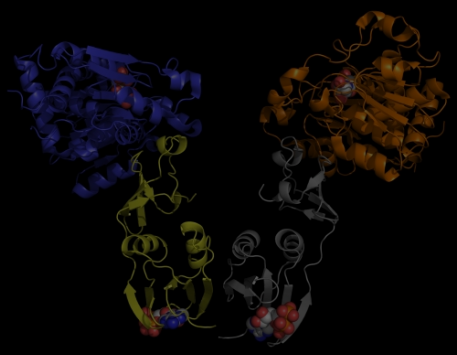

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